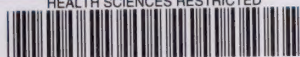


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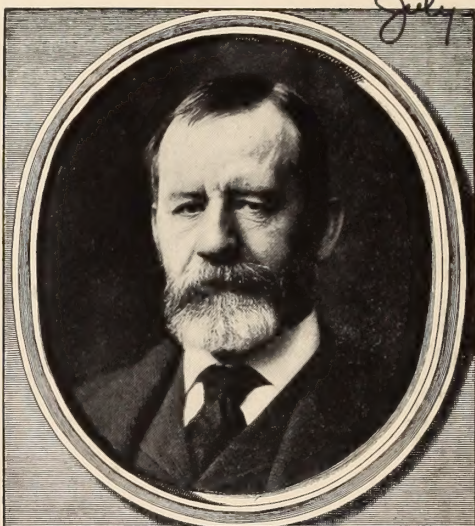
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THE  
JOURNAL OF INFECTIOUS DISEASES





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# THE BACTERIOLOGY OF ACUTE EPIDEMIC RESPIRATORY INFECTIONS COMMONLY CALLED INFLUENZA \*

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Aside from a few isolated observations on previous outbreaks of acute epidemic respiratory infections, the most important bacteriologic work on these diseases was done during the great pandemic of 1889-92. The voluminous literature that accrued from these investigations has been carefully reviewed by Finkler,<sup>1</sup> Leichtenstern,<sup>2</sup> and others. Notwithstanding the great amount of work done during this period, no observer proved that one species of bacteria was of primary importance in the causation of these diseases. The larger group of investigators working during 1890, including Weichselbaum,<sup>3</sup> Babes,<sup>4</sup> Beim,<sup>5</sup> Ribbert,<sup>6</sup> Prior,<sup>7</sup> and Finkler<sup>8</sup> in Germany and Austria, Bouchard,<sup>9</sup> Gaucher,<sup>10</sup> Vaillard,<sup>11</sup> See and Bordas,<sup>12</sup> and Saccoud<sup>13</sup> in France, and many others in widely separated localities, found streptococci and pneumococci in the sputum and postmortem material from the cases of influenza. These findings by different workers in various parts of the world during the early part of the great pandemic of 1890 led to the view that the epidemic respiratory infections were streptococcal or streptococco-pneumococcal in character. In 1890, however, Pfeiffer<sup>14</sup> announced the finding of small gram-negative bacilli in great numbers in stained smears of material from cases of influenza, and in his classic article<sup>15</sup> a short time later, he described *B. influenzae* as the primary etiologic factor in epidemic acute respiratory infections. Pfeiffer's findings were confirmed by Kitasato,<sup>16</sup> Canon,<sup>17</sup> Pfuhl,<sup>18</sup> Pfeiffer and Beck,<sup>19</sup> and others, and his contention that this organism was the causative factor in these epidemic diseases gained almost universal acceptance at this time. The previously

\* Received for publication December 4, 1916.

<sup>1</sup> 20th Century Practice of Modern Medical Science, 1898, 15, p. 3.

<sup>2</sup> Nothnagel's Encyclopedia of Practical Medicine (Am. edition), 1905, p. 523.

<sup>3</sup> Wien. klin. Wchnschr., 1890, 3, pp. 104, 123, 145, 163, 186.

<sup>4</sup> Centralbl. f. Bakteriöl., 1890, 7, pp. 233, 460, 496, 533, 561, 598.

<sup>5</sup> Ztschr. f. klin. Med., 1890, 17, p. 545.

<sup>6</sup> Deutsch. med. Wchnschr., 1890, 16, pp. 61, 301.

<sup>7</sup> München. med. Wchnschr., 1890, 37, p. 233.

<sup>8</sup> Deutsch. med. Wchnschr., 1890, 16, p. 84.

<sup>9</sup> Sem. méd., 1890, 10, p. 35.

<sup>10</sup> Ibid., p. 93.

<sup>11</sup> Ibid., pp. 39, 54.

<sup>12</sup> Compt. rend. de l'Acad. des sc. de Paris, 1890, 110, p. 197.

<sup>13</sup> Sem. méd., 1890, 10, p. 51.

<sup>14</sup> Deutsch. med. Wchnschr., 1890, 18, p. 28.

<sup>15</sup> Ztschr. f. Hyg. u. Infektionskr., 1893, 13, p. 357.

<sup>16</sup> Deutsch. med. Wchnschr., 1892, 18, p. 28.

<sup>17</sup> Ibid., 1892, 18, p. 28.

<sup>18</sup> Centralbl. f. Bakteriöl., 1892, 11, p. 397.

<sup>19</sup> Deutsch. med. Wchnschr., 1893, 19, p. 816.

described pathogenic microorganisms — streptococci and pneumococci — were henceforth considered as secondary invaders.

Pfeiffer and his associates isolated influenza bacilli from the sputum and bronchial mucus before death and from the lungs and bronchial mucosa after death of patients suffering from influenza. No observer except Canon<sup>17</sup> has satisfactorily demonstrated influenza bacilli in the blood of persons sick with the disease and Pfeiffer favored the view that influenza was a local infection only, an invasion of the blood by the bacilli not occurring under ordinary circumstances. Experiments designed to produce the disease in animals, including mice, rats, guinea-pigs, rabbits, swine, dogs, and cats, were uniformly negative. In monkeys in a few instances very slight general reactions followed the inoculations, which Pfeiffer considered significant. The characteristic lesions, however, were never found in these animals and when death occurred the cause was probably toxic in nature. The animal experiments of Klein,<sup>20</sup> Kruse,<sup>21</sup> and others, yielded negative results. The failure to demonstrate influenza bacilli in the blood of patients suffering from the disease, and the failure to reproduce the disease in animals, left Pfeiffer's conclusions open to criticism and subsequent bacteriologic results have failed to yield proof of his contentions.

In recent years von Jaksch,<sup>22</sup> Sacquepee,<sup>23</sup> Kleinberger,<sup>24</sup> Bezançon and Israëls de Jong,<sup>25</sup> Lord,<sup>26</sup> Davis,<sup>27</sup> and others, in studies of isolated epidemics of influenza have failed to find influenza bacilli with any degree of regularity, but have found streptococci, pneumococci, and other organisms in predominating numbers. Furthermore, during attacks of various acute infectious diseases influenza bacilli have been observed in the mouth as common associates with other bacteria. Thus Elmassian,<sup>28</sup> Davis,<sup>29</sup> Jochmann,<sup>30</sup> and others have found these organisms in the sputum in lobar pneumonia, bronchopneumonia, chronic bronchitis, tuberculosis, whooping cough, measles, and many other diseases. Hence, tho Pfeiffer's work is a classic piece of bacteriologic investigation, his conclusions pertaining to the pathogenic importance of the influenza bacillus are shown by the foregoing observations to have been unwarranted. This microorganism more likely is a common inhabitant of the upper respiratory tract in a great number of acute infectious diseases and except in influenzal meningitis, is of little etiologic significance.

During the winter of 1915-16 a widespread epidemic of acute respiratory infections occurred in the United States which resembled in every detail epidemic influenza. The outbreak was at its height during December, 1915, and January, 1916, and the general mortality of the country was greatly increased. This disease, different from the common acute rhinitis, can be described as an acute erysipelatous

<sup>10</sup> Ann. Rep. of the Med. Officer of the Local Govt. Board, London, 1891-92.

<sup>21</sup> Deutsch. med. Wchnschr., 1894, 20, p. 513.

<sup>22</sup> Berl. klin. Wchnschr., 1899, 36, p. 425.

<sup>23</sup> Arch. de méd. expér. et d'anat. path., 1901, 13, p. 562.

<sup>24</sup> Deutsch. med. Wchnschr., 1905, 36, p. 575.

<sup>25</sup> Reviewed in Bull. de l'Inst. Pasteur, 1905, 3, p. 372.

<sup>26</sup> Publ. Mass. Gen. Hosp., 1908-09, 2, p. 715.

<sup>27</sup> Jour. Am. Med. Assn., 1915, 64, p. 1814.

<sup>28</sup> Ann. de l'Inst. Pasteur, 1899, 13, p. 621.

<sup>29</sup> Jour. Am. Med. Assn., 1907, 48, p. 1563; 1915, 64, p. 1814; Jour. Infect. Dis., 1913, 12, p. 386.

<sup>30</sup> Lubarsch u. Ostertag Ergebnisse der Allg. Path., 1909, 13 (Abt. 1), p. 107.



inflammation of the mucosa of the upper respiratory passages or of the intestinal tract, with frequent complications and sequelae that often endangered the life of the individual. Because of the unsettled state of our knowledge of the etiology of such diseases and because of their great importance from the standpoint of public health it was thought advisable to study the bacteriology of this epidemic as it occurred in Chicago. The results of this work, partially detailed in a preliminary report,<sup>31</sup> form the basis of this communication.

#### TECHNIC

Cultures were made of material from the upper respiratory passages and the blood of patients at various times during the course of the infection. The material from the nose, throat, and pharynx was collected in sterile containers and washed thoroughly through several changes of sterile normal salt solution. Cover-glass preparations of the fresh material were stained for ordinary bacteria and tubercle bacilli. Aerobic and anaerobic cultures were made on human-blood agar, ascites dextrose broth, ascites dextrose agar, and Loeffler's serum media and incubated at 35-37 C. for 1-14 days. Also in many instances sputum cultures were made by the intraperitoneal inoculation of mice with suspensions of fresh sputum. For blood cultures the blood was drawn from the median basilic vein, and various amounts of whole blood were added to plain agar, plain broth (0.7% acid to phenolphthalein), ascites dextrose agar, and ascites dextrose broth. All cultures were allowed to incubate 14 days before they were discarded. The bacteria were identified by the usual bacteriologic methods. The virulence of the various strains of streptococci was determined in a number of instances. For these tests young rabbits were used and varying doses of a 24-hour broth culture of the streptococcus were injected intravenously. The biologic groups of the pneumococci isolated in this work were determined by agglutination tests with the specific sera of Groups I and II.

In the filtration experiments Berkefeld filters of the 'N' type and porcelain filters of the Maussen type were used. The nasal discharge or sputum was macerated in a small amount of sterile salt solution before filtering. Cultures of the filtrates were made in ascites fluid containing a sterile piece of rabbit kidney, as well as on the media previously described for cultures of the original material.

Bacteriologic examination has been made of the sputum, nasal discharge, and pharyngeal mucus from 61 cases of acute epidemic respiratory infection during the acute stage of the disease, and the results are tabulated in Table 1. In 46 instances a virulent hemolytic streptococcus was isolated from this material and in 6 of these this organism was found in pure culture. In 30 cases a green-producing streptococcus was obtained, but only once in pure culture. Pneumococci were isolated in 30 instances, in 4 of which the cultures were pure. The influenza bacillus was found in only 1 instance, and then in mixed culture

<sup>31</sup> Mathers, Jour. Am. Med. Assn., 1916, 66, p. 30.

with a hemolytic streptococcus. In previous work it had been observed that mouse-inoculation of cultures of pneumonic sputum occasionally yielded influenza bacilli as well as pneumococci. This method of culture was also used in 22 instances, with uniformly negative results as far as influenza bacilli were concerned; the findings in every instance corresponded very closely with those of the other cultural methods. Staphylococci were found in 51 instances and many of the strains were hemolytic. Other organisms occasionally found in the aerobic cultures were *Micrococcus catarrhalis*, *B. pneumoniae*, and various types of diphtheroid bacilli. Anaerobic cultures usually yielded *B. fusiformis*, *Staphylococcus albus*, and diphtheroid bacilli. Green-producing streptococci were also observed in many of the anaerobic cultures, and in one instance an organism similar in morphology and cultural charac-

TABLE 1  
THE BACTERIOLOGIC FINDINGS IN 61 CASES OF ACUTE EPIDEMIC RESPIRATORY INFECTION

Organism	Number of Pure Cultures	Number of Mixed Cultures	Total Number of Cases	Percentage of Total Number of Cases
Hemolytic streptococcus .....	6	40	46	75.4
<i>S. viridans</i> .....	1	29	30	49.1
<i>Pneumococcus</i> ..	4	26	30	49.1
<i>B. influenzae</i> ....	0	1	1	1.6
<i>M. catarrhalis</i> ..	0	6	6	9.8
<i>B. pneumoniae</i> ..	0	1	1	1.6
<i>S. albus</i> .....	0	51	51	83.6

teristics to *B. rhinitis* described by Tunnicliff<sup>32</sup> was found in the nasal discharge. In 9 cases blood cultures were made during the height of the disease. Four of these were positive, 3 yielding hemolytic streptococci and 1, pneumococci in pure culture. The cultures from the nasal discharge, during the first 2 or 3 days of the disease, were often negative, while those from the pharynx and throat yielded hemolytic streptococci in predominating numbers. After the second or third day, however, hemolytic streptococci were usually found in the nasal discharge also. Pneumococci were found in the nasal discharge only twice.

In 3 instances cultures were made of filtrates of the nasal discharge and sputum during the first 3 days of the disease. Both Berkefeld 'N' filters and Maussen porcelain filters were used. All cultures of filtrates were negative. In cultures on anaerobic ascites-fluid tissue media, after 3-5 days, there appeared a fine turbidity around the tissue, but similar

<sup>32</sup> Jour. Infect. Dis., 1913, 13, p. 283; 1915, 16, p. 493.

changes appeared in the control tubes and probably represented modifications in the media caused by changes in the tissue. No living bacteria or virus could be demonstrated.

In 46 instances a hemolytic streptococcus was found to be the predominating organism in the nasal discharge and sputum of these cases of acute epidemic respiratory infection. In 3 instances it was found in the blood stream in pure culture. The cultural characteristics of the different strains were very similar. On standard blood-agar plates (1 c.c. human blood to 9 c.c. of plain agar), the colonies were small, slightly moist, grayish-white, and surrounded by a sharply defined clear zone of hemolysis, 1-3 mm. in diameter. Growth in ascites dextrose broth was marked, a coarse turbidity and granular sediment appearing in the media after 18-24 hours. In plain broth the growth was scant, and litmus milk was acidified and usually coagulated. Dextrose, saccharose, lactose, and salicin were the most common carbohydrates to be changed. A few strains fermented raffinose and a few mannite, but in no instance was inulin fermented. The virulence for rabbits of 16 strains of the hemolytic streptococcus was studied, and it was found that 1 c.c. of a 24-hour plain-broth culture usually produced arthritis and death in 3-21 days. Morphologically, these hemolytic streptococci varied somewhat, but usually they were medium-sized, round or oval, and gram-positive, growing in pairs and short chains in liquid media. In no instance were capsules demonstrable.

The streptococci were usually found early in the course of the disease, and if no complications appeared they decreased in number or disappeared with the decline of the infection. Cultures from 7 cases made 10 days to 3 weeks after the onset of the disease yielded virulent hemolytic streptococci only once. Green-producing streptococci were the predominating organisms late in the course in all these cases.

In 30 cases green-producing streptococci were isolated from the nose and throat. These grew on standard blood-agar plates as small round greenish colonies surrounded by a narrow green halo. Morphologically, they were medium-sized, oval or oblong, gram-positive, and arranged in pairs and short chains. The different strains acidified litmus milk in varying degrees, grew sparingly in plain broth, and fermented dextrose, saccharose, lactose, and salicin usually, but variations were noted in mannite, inulin, and raffinose. The virulence for rabbits was tested in 6 instances and all the strains were found to be of very low virulence.



Pneumococci were also isolated in 30 instances from the nose and throat in these cases, and once from the blood stream. The pneumococci were often atypical in their cultural characteristics. Their biologic reactions have been tabulated in Table 2. They were found to belong usually to Group I or Group IV. The organisms of Group IV predominated, but not to such an extent as to allow any important deductions, for these two types were predominant in the lobar pneumonias occurring in Chicago during the winter of 1915-16. Group I, however, exceeded Group IV in the typical pneumonias, a condition just the reverse of that found in the acute respiratory infections (Table 2).

TABLE 2

THE BIOLOGIC CLASSIFICATION OF THE DIFFERENT STRAINS OF PNEUMOCOCCI FOUND IN THE EPIDEMIC RESPIRATORY INFECTION AND LOBAR PNEUMONIA DURING THE WINTER OF 1915-16

Group	Epidemic Respiratory Infection		Lobar Pneumonia	
	Number of Cases	Percentage of Total	Number of Cases	Percentage of Total
I	10	33.3	14	50.0
II	6	20.0	5	17.8
III	1	3.3	1	3.5
IV	13	43.4	8	28.7

Of the other organisms found in these cultures little need be said. The strain of influenza bacilli and the strains of *Micrococcus catarrhalis* were typical culturally and morphologically. Many of the staphylococcus strains were hemolytic when first isolated.

In 3 cases the opsonins, agglutinins, and complement-fixing bodies were studied at various stages of the disease. In 2 instances there was a marked increase in the opsonins from the 6th to the 8th day of the infection for the four strains of virulent hemolytic streptococcus tested. There was no demonstrable increase in the agglutinins or complement-fixing bodies in either of these cases. The opsonins also rapidly declined with the recovery of the patient.

The importance of these results from the standpoint of the etiology and pathology of acute epidemic respiratory diseases may be discussed briefly. The clinical and pathologic characteristics of influenza are suggestive of a streptococcal infection. Inflammation of the mucosa of the upper respiratory tract simulating erysipelas in its appearance and mode of spreading, general lymph-node enlargement, the marked

tendency to attack the serous surfaces of the body, as well as the acute course, marked prostration, and characteristic complications, are all stigmata of a streptococcic disease. In this work hemolytic streptococci were the significant finding in most instances. These streptococci were markedly hemolytic and highly virulent for rabbits, resembling in every detail the streptococcus associated with scarlet fever and epidemic sore throat as described by Davis<sup>33</sup> and others. This organism has been called the beta type of hemolytic streptococcus by Smith and Brown.<sup>34</sup> These virulent organisms are rarely found in the mouths of normal individuals, but are commonly associated with acute infectious diseases in which there are acute inflammatory processes in the upper respiratory tract. Moreover, it is a striking fact that during this epidemic the incidence of other acute infectious diseases, especially scarlet fever and measles, was greatly increased and atypical exanthematous complications were very commonly observed. Also many cases were very similar in every way to epidemic tonsillitis. Such occurrences strongly suggest the possibility of a close relation between epidemic respiratory infections, scarlet fever, and epidemic sore throat. Altho *Streptococcus viridans* and the pneumococcus must be mentioned as possible etiologic factors in the infection, their similarity to the organisms normally present in the mouth favors the inference that they are of secondary importance. It seems more probable that the etiologic factor was a virulent hemolytic streptococcus. At least it is certain that the influenza bacillus was not of any etiologic significance in the epidemic of acute respiratory infections occurring in Chicago during the winter of 1915-16.

#### CONCLUSIONS

During the winter of 1915-16 an epidemic of acute respiratory infection occurred in Chicago which closely resembled in its clinico-anatomical manifestations and epidemiology so-called true influenza as it appeared in 1889-92.

In a bacteriologic study of material from 61 cases of this disease the influenza bacillus was found in only 1 instance and then in small numbers. On the other hand, virulent hemolytic streptococci similar in cultural characteristics and virulence to the streptococcus commonly associated with epidemic sore throat and scarlet fever were found in

<sup>33</sup> Jour. Am. Med. Assn., 1912, 58, p. 1852.

<sup>34</sup> Jour. Med. Research, 1914, 31, p. 455.

the nose, throat, and pharynx in 46 and in the blood in 3 cases of this epidemic disease.

*Streptococcus viridans* and the pneumococcus were each found in 30 instances, these organisms in virulence and biologic characters closely resembling the organisms found in the normal mouth.

The predominance in the discharges from the nose, throat, and pharynx of patients suffering from the epidemic respiratory infection of a virulent hemolytic streptococcus not usually found in the normal mouth, the absence of the influenza bacillus, and the character of the associated pathologic changes suggest that this disease was caused by a virulent hemolytic streptococcus.



# A LEPTOTHRIX ASSOCIATED WITH CHRONIC BRONCHOPNEUMONIA \*

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There are described in the literature cases of chronic pulmonary infection simulating tuberculosis in which tubercle bacilli were not found after repeated search and in which the tuberculin reaction was negative. There is considerable confusion in the nomenclature of these cases due in large part to the fact that the etiology is difficult to establish.

Thus cases of so-called pseudotuberculosis are described in which diphtheroid organisms have been isolated or in which the bacteriology has not been worked out. Another source of confusion is the variety of names applied to the filamentous organisms found associated with the infections. Wright<sup>1</sup> describes such infections under the head of nocardiosis and later<sup>2</sup> groups them under the term streptothricosis from streptothrix, a name sometimes applied to the whole group of filamentous organisms. Most authors follow a classification similar to that given by Jordan:<sup>3</sup>

Trichomycetes	{	Leptothrix—no branching
		Cladothrix—false branching
		Streptothrix—true branching, spores
		Actinomyces—true branching, no spores

There have been a number of infections of the lung in which streptothrix-like organisms were isolated in pure culture from the sputum. Claypole,<sup>4</sup> in a number of these cases, made skin tests similar to von Pirquet's, using preparations of 2 varieties of streptothrix analogous to Koch's old tuberculin. Specific reactions were obtained.

Pulmonary infections with leptothrix are rare. Leyden and Jaffe<sup>5</sup> describe such organisms in stains of the sputum of putrid bronchitis, but no growths were obtained. Kato<sup>6</sup> reports a pleuropneumonia which

\* Received for publication December 11, 1916.

<sup>1</sup> Osler's Modern Medicine, 1907, 1, p. 340.

<sup>2</sup> Ibid., 1913, 1, p. 1045.

<sup>3</sup> General Bacteriology, 1910.

<sup>4</sup> Arch. Int. Med., 1914, 14, p. 104.

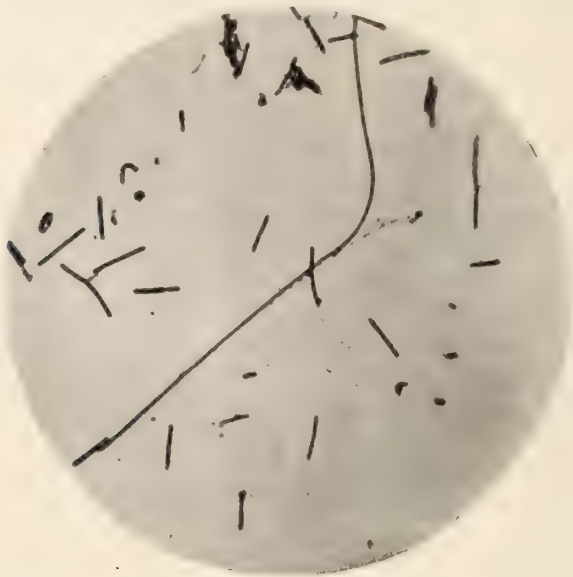
<sup>5</sup> Deutsch. Arch. f. klin. Med., 1867, 2, p. 488.

<sup>6</sup> Mitt. a. d. Med. Fakult. d. k. Univer. zu Tokyo, 1915, 13, p. 441.

they believe to have been caused by a leptothrix. The patient presented a clinical picture similar to that described here. The leptothrix isolated in pure culture was nonacidfast. The history of our case follows.

The patient was an unmarried American woman, aged 49 years. She had had typhoid fever at 14 years, but no other illness so far as she could remember. She gave no history of association with tuberculous persons.

About 1 month before admission to the hospital, the patient had caught cold and had had a chill followed by sharp pain in the left side of the chest and cough. The sputum was profuse, thick, and of a dark color. Later it became thin, frothy, and colorless except for yellow flakes. The pain diminished in intensity and frequency.



The leptothrix associated with chronic bronchopneumonia.  $\times 1200$ .

The patient was somewhat emaciated. The respiratory excursion was diminished on the right side of the chest. Vocal fremitus was decreased over the lower lobe of the right lung. The percussion note over the right back was dull at the middle of the scapula, the dullness increasing toward the base where it merged into flatness. There was no sharp line marking the beginning of this dullness and it did not shift on change of the patient's position. The breath sounds over the upper part of the dull area were bronchial, but louder in some places than in others. At the base they were suppressed. Mucous and submucous râles were heard over the lower right chest behind and fewer over the lower left back.

The skiagraph of the chest showed a diffuse shadow over the lower right lobe with here and there more intense shadows 1-3 cm. in diameter. The left border of the heart was 10 cm. from the mid-sternal line. There was a faint soft systolic murmur heard at the apex and transmitted somewhat

to the left of the area of cardiac dulness. There was a slight accentuation of the pulmonic second sound.

The blood showed 4,952,000 erythrocytes; 11,350 leukocytes; 76% (Sahli) hemoglobin. The systolic blood pressure was 125 mm. A Wassermann test of the blood serum was negative.

The urine had a specific gravity of 1.007. Albumin +. No casts.

The patient had a fever for a month after entering the hospital. The temperature was irregular, higher in the afternoon than in the morning, and varied from 97 to 100.6 F. The physical signs changed gradually. The dullness became less marked, the râles diminished in number, and in 2½ months were no longer heard. The breathing lost its bronchial character. Some dullness, however, remained as long as the patient was under observation, tho she had regained her weight and felt well.

The sputum was examined repeatedly by the antiformin method for tubercle bacilli, but none were found.

Cultures of the sputum were made on blood-agar slants aerobically and anaerobically. The aerobic cultures yielded *Streptococcus viridans*, *Micrococcus catarrhalis*, and some small gram-negative organisms resembling diphtheria bacilli in morphology, but very slender.

The anaerobic cultures showed many colonies of gram-positive organisms growing in filaments and bacillary forms. The organisms were found plentifully in direct smears of the sputum. The pure cultures when stained with carbolfuchsin and decolorized with 10% hypochloric acid in 95% alcohol were for the most part decolorized, but sections of filaments and granules in the filaments retained the fuchsin so that smears counter-stained with methylene blue showed the long blue filaments with red dots and dashes spaced irregularly. Some of the short forms retained the fuchsin and others did not. In direct smears of the sputum the organisms were nonacidfast. They grew fairly well in anaerobic cultures on blood agar. The colonies developed in 5-6 days as small translucent flat discs, reaching a maximum of 1 mm. in diameter. In subsequent transfers growth appeared at the end of 48 hours, and at the end of 5 or 6 days the blood agar had become brown. In anaerobic dextrose ascites broth the organism tended to form colonies, which sank to the bottom of the medium. There was no growth on ordinary media and none aerobically.

A skin test was made with a suspension of the killed organisms. On account of the difficulty of applying enough material by the cutaneous method an intracutaneous injection was used similar to the intracutaneous tuberculin test of Mantoux. By inoculation of 3 individuals free from pulmonary infection with graded doses of killed organisms it was found that 0.1 c.c. of a suspension of approximately 500,000,000



organisms per c.c. produced no reaction. This amount was injected intracutaneously over the outer aspect of the upper arm of the patient, and 0.1 c.c. of a 1 : 5000 solution of old tuberculin injected in the same way in the other arm. The tuberculin, which was in an amount recommended by Mantoux and others, produced no reaction, while the leptothrix injection was followed by redness and induration, which was well marked from 24 to 48 hours following the injection, subsiding in about 3 days.

Rabbits injected intravenously with the organism either died overnight, when a large dose was given, or were not affected at all. No experimental pulmonary infection was obtained.

The morphology of the leptothrix is shown in the photomicrograph.

# INTRAVENOUS INJECTION OF TYPHOID VACCINE \*

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A small series of cases of typhoid fever treated with intravenous injections of typhoid vaccine have been studied especially with respect to the changes in temperature, the leukocytes, the agglutinin and opsonin, the blood pressure, and the coagulation time. Observations were made several times each day. The vaccine used was a sterile suspension of typhoid bacilli heated at 60 C. for half an hour on 2 consecutive days, to which had been added 0.5% phenol.

## CASE 1

The patient, a man 23 years of age, had been sick for 5 days with continuous fever; spleen palpable; pulse dicrotic; rose spots present. On the 15th day, the fever being still continuous, 20,000,000 typhoid bacilli were injected intravenously. Thirty minutes later the patient had a severe chill for 20 minutes; the temperature rose to 105.4, reaching its maximum 8 hours after the injection; in 10 hours it had fallen to 96. For the next 6 days fever of intermittent type ran between 100 and 102 and then the temperature fell to normal. The day after the injection the condition was much improved and convalescence set in with a rapid gain in weight and strength.

The leukocytes for 10 days preceding the injection of the vaccine averaged 5000 per cubic centimeter, 60% polymorphonuclears, 40% mononuclears. Four hours after the injection the count dropped from 4300 to 1500; in 18 hours it rose to 12000 and 24 hours later it fell to 5000, where it remained. At the initial drop the polymorphonuclears fell to 12% but increased rapidly, reaching 89% in 12 hours, and returning to the normal proportion in 36 hours.

The blood pressure fell from 120 to 105 after the injection and returned to 120 in 2 days.

The coagulation time showed no change during the reaction period (Boggs coagulometer). For agglutinin and opsonin see Chart 1.

## CASE 2

The patient, a man 23 years of age, entered the hospital on the 5th day of illness, very sick and toxic; temperature 105, respirations 30, and leukocytes 4000. For the next 6 days his condition remained about the same. On the 11th day he had a moderate intestinal hemorrhage. On the 12th day 20,000,000 typhoid bacilli were injected. Twenty-five minutes later he had a severe chill for 20 minutes, with a rise in temperature from 103 to 105, rapid pulse and respiration, and a diffuse erythema. Three hours later a severe epistaxis, bleeding from the gums, and a small intestinal hemorrhage occurred, and

\* Received for publication, December 15, 1917.

morphin and horse serum were given. In 18 hours the temperature dropped to 96, pulse to 80, respiration to 20, and the general condition improved; there was a general eruption of rose spots. The following 9 days the temperature ranged from normal to 102.5. On the 22nd day 40,000,000 typhoid bacilli were injected. This was followed by a chill, rise in temperature to 105.4 and fall to normal in 12 hours. From this time on, the temperature remained normal and prompt convalescence took place.

For several days preceding the injection the leukocytes were approximately 4000, with 60% polymorphonuclears and 40% mononuclears. In 4 hours after the injection they fell to 1900, with a decrease in polymorphonuclears to 18%;

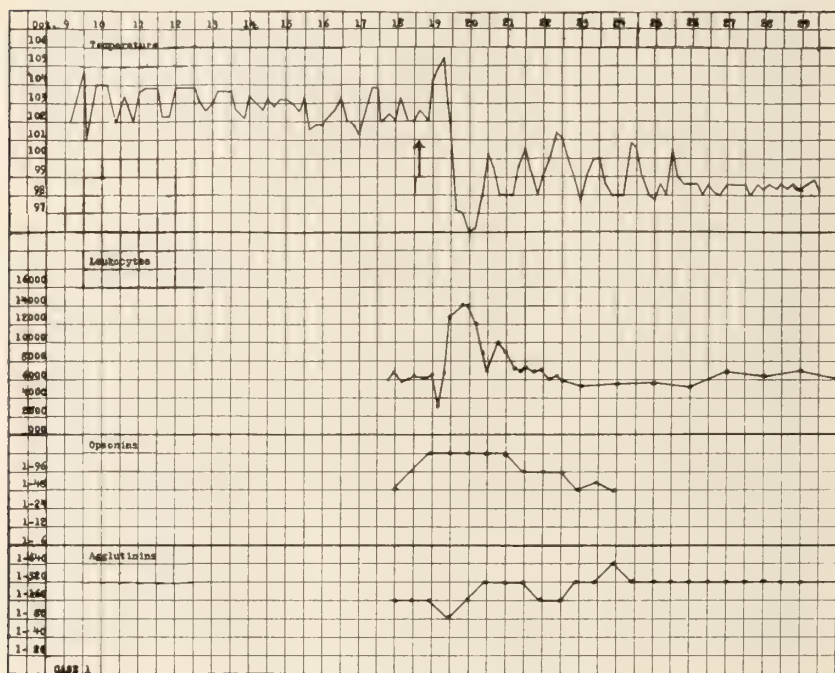


Chart 1.—Agglutinin, Opsonin, etc., Case 1.

in 12 hours the leukocytes increased to 16000, the polymorphonuclears to 88%; in 24 hours the count was 5000. Following the second injection the same initial drop occurred followed by a leukocytosis of 14800, polymorphonuclears 88%; in 36 hours the count was 5000.

For agglutinin and opsonin see Chart 2.

The blood pressure fell from 110 to 100 during the period of reaction and in 8 days gradually reached 130. At the second injection it fell to 120, returning to 130 the next day.

The coagulation time showed a slight increase during the chill from 3 minutes to 2 minutes.

## CASE 3

A man, 27 years of age, entered the hospital on the 8th day with a fever of 100 to 103, palpable spleen, rose spots, and agglutinin in serum. On the 12th day of illness 20,000,000 bacilli were given intravenously; this was followed by only a slight chill, nausea and vomiting, and a rise in temperature from 101 to 103. The temperature continuing unchanged, on the 15th day a second injection of 40,000,000 was given. This was followed by a chill, rise in temperature to 100, succeeded by a drop in 12 hours to 97. The temperature then rose again and remained at from 99 to 102.4. On the 23rd day the patient was given a third injection of 80,000,000. This was followed by a severe chill and a rise in temperature to 106 with fall in 12 hours to 97.8, whereupon the temperature remained normal for 17 days. At this time the patient had a typical relapse which continued for a week.

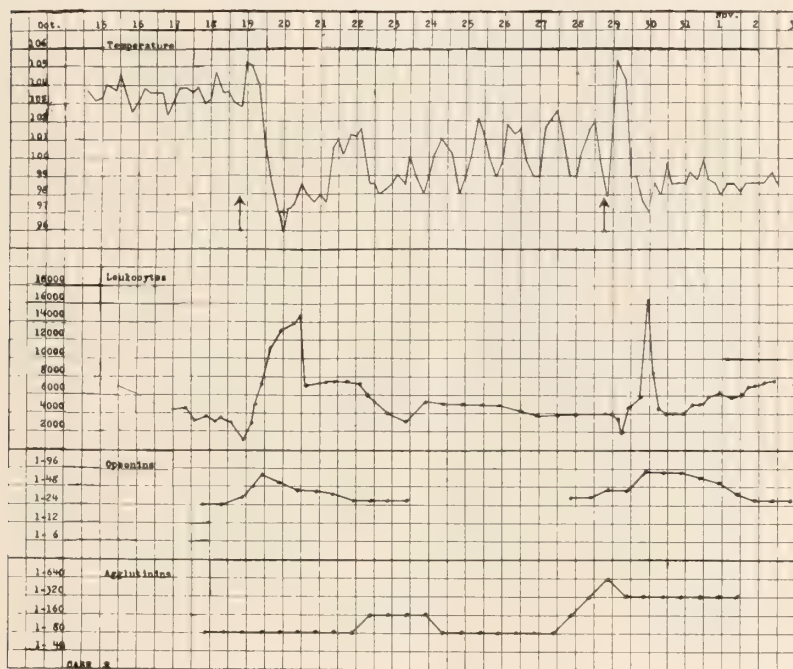


Chart 2.—Agglutinin, Opsonin, etc., Case 2.

After each injection the blood pressure dropped from 124 to 98. After the first injection there was no fall in the number of leukocytes, but in 4 hours it rose to 11400 with a relative increase in the polymorphonuclears. The second and third injections produced a leukocytosis of 24000 for 36 hours without any initial drop. If an initial drop occurred in this case, it must have taken place earlier than the fourth hour, when the count was made.

For agglutinin and opsonin see Chart 3.



## CASE 4

A woman, 30 years of age, entered the hospital on the 5th day of illness with all signs and symptoms of typhoid fever. Blood cultures were positive and typhoid bacilli were isolated from the stool and the urine.

On the 10th day the patient was given intravenously 40,000,000 bacilli; 20 minutes later she had a severe chill, which continued for 30 minutes, during which cyanosis developed; pulse 120; respiration 36. Eight hours later it rose again to 102.4 and assumed a remittent type. Otherwise the vaccine had little effect on the course, the temperature subsiding by lysis in 3 weeks.

The leukocytes, following the vaccine injection, dropped in 3 hours from 5000 to 2200, and then rose rapidly to 27000.

Agglutinin and opsonin determinations were not made.

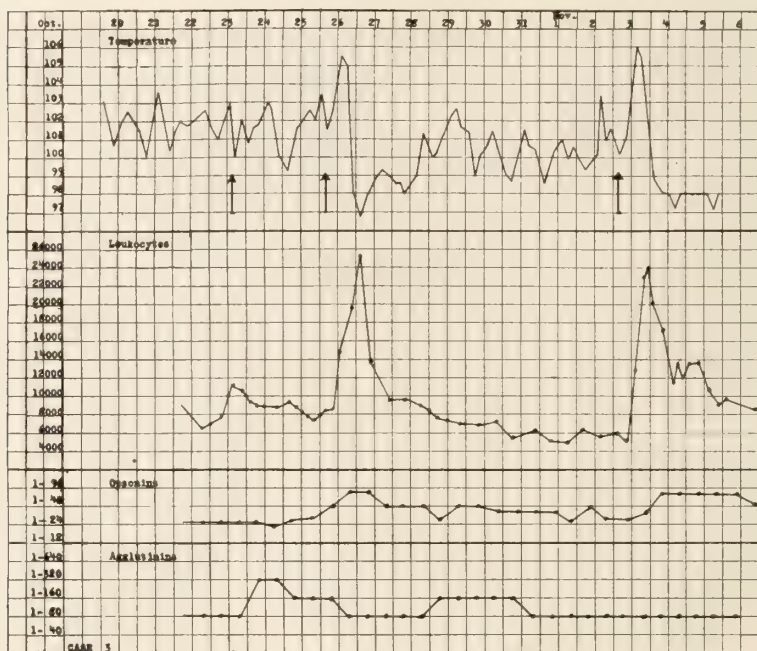


Chart 3.—Agglutinin, Opsonin, etc., Case 3.

The coagulation time, tests being made hourly beginning 2 hours before, and terminating 13 hours after, vaccine injection, fell from an average of 3 minutes to an average of 2 minutes during the reaction (Bogg's coagulometer).

## CASE 5

A man, 21 years of age, entered the hospital about the 7th or 8th day of illness with a continuous temperature, leukocyte count 8000, positive blood culture and agglutinin test. The patient was very toxic and delirious. On the

12th day of illness he was given 40,000,000 typhoid bacilli. A chill followed and a rise in temperature to 106.6, succeeded by a fall in 88 hours to 97 and a return to between 102 and 105. The general condition was not improved and death took place on the 16th day. The blood pressure before the injection was 100; afterward it dropped to 78 and remained at this until death.

The leukocytes decreased slightly from 7000 and then rose in 12 hours to 19000 and increased gradually for 2 days to 22000 at the time of death.

(For agglutinin and opsonin see Chart 4.)

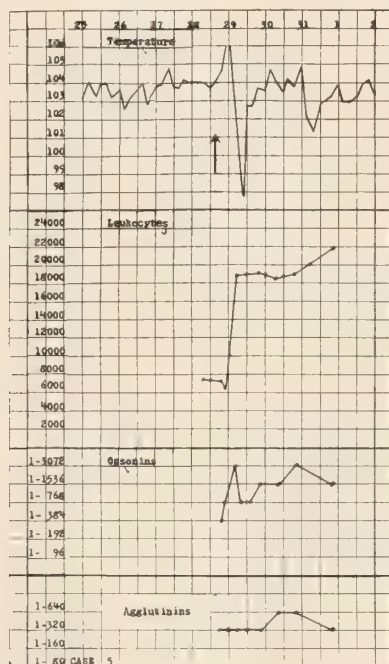


Chart 4.—Agglutinin, Opsonin, etc., Case 5.

#### CASE 6

A man, 20 years of age, entered the hospital on the 7th or 8th day of illness with a continuous fever, rose spots, palpable spleen, leukopenia, positive agglutination test and blood culture.

About the 13th day of illness 40,000,000 bacilli were given intravenously. This was followed by a typical reaction, but after the initial drop the temperature curve continued practically unchanged assuming a remittent type and falling by lysis. The condition generally, however, was distinctly improved.

The leukocytes made the characteristic drop, rose to 13000, in 10 hours fell to 7000, and then gradually to 5000.

For agglutinin and opsonin see Chart 5.

For purposes of comparison a perfectly normal man, 21 years old, was given 40,000,000 typhoid bacilli intravenously. In 30 minutes a severe chill

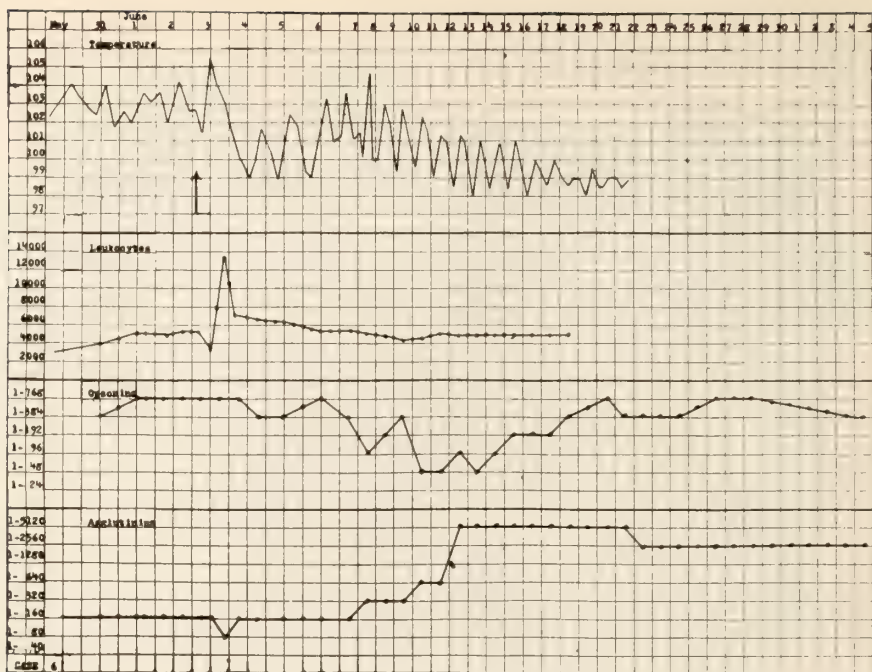


Chart 5.—Agglutinin, Opsonin, etc., Case 6.



Chart 6.—Opsonin, Agglutinin, etc., Normal Man.

lasting 20 minutes came on, with a rise in temperature to 104.4 followed by gradual subsidence to normal in 48 hours. Seven days after the injection the spleen was just palpable and remained so for 5 days. On the 9th day after the injection the serum gave agglutination at 1:80 and remained at this titer for some time. The opsonin increased slightly the 2nd day (see Chart 6).

The leukocytes rose in 24 hours to 27000 and gradually declined in 3 days to 8000.

In all the cases the immediate effect of the vaccine was a chill for 20-30 minutes, followed by a rapid rise of temperature, which reached the maximum in 3-5 hours and then fell again in the course of the next 18 hours or so. The chill was accompanied by an increased rapidity of pulse and respiration and sometimes cyanosis. When the temperature fell, there was usually profuse perspiration.

In 1 case the temperature became remittent after 1 injection, in 2 cases after 2 or 3 injections; in 3 cases which received only 1 injection the fever continued, but changed from continuous to remittent, and here there was little or no effect on the course of the attack.

The leukocytes were counted every 4 hours just before, and after the injection of the vaccine. Before the injection the counts usually ranged from 4000 to 5000, polymorphonuclears 60% and mononuclears 40%; 4 hours or so after the injection the count usually was 1500 to 2000, polymorphonuclears 18-20% and mononuclears 78-80%; some 12 hours or so later the count usually had increased from 12000 to 20000, polymorphonuclears 80-88%.

The leukocytosis in the healthy man after injection of vaccine indicates that the leukocytosis in the typhoid patient after the injection of vaccine is not altogether specific as claimed by Gay and Chickering.<sup>1</sup> McWilliams<sup>2</sup> was unable to obtain any evidence of a specific hyperleukocytosis from typhoid vaccine in rabbits.

In determining the concentration of agglutinin and opsonin, we used the active serum in Cases 1, 2, and 3, and also in the case of the normal man, and in Cases 5 and 6, the heated serum. To determine the agglutinin extinction we used the macroscopic method, the tubes being incubated for 2 hours at 36 C. and read the following morning. To estimate the opsonin we used the extinction method, incubating the tubes for 7 minutes in the case of the active serum and for 25 minutes in the case of the heated serum. In 6 normal persons we found that the titer of the normal typhoid opsonin did not exceed 1-24. In most of our cases the titer of the agglutinin increased somewhat in 5-10 days

<sup>1</sup> Arch. Int. Med., 1916, 17, p. 303.

<sup>2</sup> Jour. Immunol., 1916, 1, p. 159.



after the injection, and the opsonin also increased, sometimes falling again, and there seemed to be no parallelism between the course of the agglutinin and the opsonin. The results of our observations on the concentration of the agglutinin and opsonin in the serum of typhoid patients injected intravenously with typhoid vaccine do not appear to lend any direct support to the view that the recovery or improvement observed in some cases is caused by a rapid increase in the formation of antibodies or rapid discharge into the blood of antibodies already produced.

As a rule, the blood pressure after the injection decreased 10-15 mm., gradually returning to the previous standard in a few days. The blood pressure was subnormal in all our cases and the least change resulted in those patients whose pressure was 100 or less.

Except in Case 4, only slight changes were observed in the coagulation time.

The results of the intravenous injection of the vaccine on the course of the disease in our cases may be summarized as follows:

One case was apparently aborted; 3 seemed distinctly improved and their course shortened; while 2 were unaffected, the patient in one of these dying.

#### SUMMARY

The immediate results of the intravenous injection of typhoid vaccine, such as chill, rise and fall of temperature, leukocytosis, and changes in the concentration of agglutinin and opsonin, usually in the direction of an increase, were the same in the normal man as in the typhoid patient, and our results do not support the view that the reaction is essentially specific. Except in so far as our results show that leukocytosis is rather constant after the injection of vaccine, they do not appear to support any particular view advanced to explain the action of intravenous injection of foreign protein in infectious diseases. The number of cases observed by us is too small to allow any conclusions as to the therapeutic effect of typhoid vaccine in typhoid fever, but the results obtained would seem to correspond fairly well with the results obtained in larger series.

## AGGLUTINATION IN TYPHUS FEVER \*

GEORGE BAEHR

*From the Pathological Laboratory of the Mount Sinai Hospital, New York*

The observation has been made by Olitsky<sup>1</sup> that specific agglutinins, precipitins, complement-fixing antibodies, and opsonins for *B. typhi-exanthematici* (Plotz) are regularly present in the blood of persons convalescent from typhus fever. He reported that "agglutinins (and other antibodies) are usually absent at the height of the disease, but increase in amount at the crisis, until they reach their maximum well along in the apyrexial period."

In the course of further investigations on typhus fever carried on in Volhynia, Russia, during the first 3 months of 1916, at the invitation of the Austro-Hungarian Government, it became necessary to make similar agglutination studies. It has been possible to confirm the observations of Olitsky on much greater material, and to ascertain in more detail the course of the development of antibodies in this disease.

In preparing the tests, the microscopic method was used. The relatively slow growth of the organism and its tendency to clump on standing made the macroscopic method of agglutination impractical. The microscopic tests with this organism, as Olitsky has pointed out, are usually more conservative than the macroscopic.

The technic employed has previously been described. Readings were made after 1 hour, at room temperature, and only a definite agglutination into clumps, with complete cessation of brownian movement, was considered positive. When the clumps were large and there were no free bacteria to be seen between them, the reaction was considered ++. The lowest dilution used was 1:50.

Altogether, 271 agglutination tests were made on the serum of 100 persons with typhus fever. In 46 of the patients, from 3 to 8 examinations were made at intervals during the illness and early convalescence.

\* Received for publication December 17, 1916. Work done under the tenure of a George Blumenthal Jr. fellowship in pathology.

These investigations on the typhus fever of Eastern Europe were carried on under the auspices of the American Red Cross by an expedition, consisting of Dr. H. Plotz and the author, which was sent out by the Mount Sinai Hospital. A report of the blood-cultures and a preliminary communication of the studies in prophylactic immunization in Serbia, Bulgaria, and Volhynia, Russia, have already been published (*Jour. Am. Med. Assn.*, 1916, 67, p. 1597; *Jour. Infect. Dis.*, 1917, 20, p. 201).

<sup>1</sup> *Jour. Infect. Dis.*, 1915, 17, p. 20.

The rest were only examined on one or two occasions. Agglutinins were demonstrable in 43 of the 46 cases studied systematically, or in 93%.

Although Olitsky had already reported that agglutinins in dilutions greater than 1:50 are never found in the blood of persons who have not had typhus fever, nor have been exposed to the disease, 28 non-typhus cases were examined as controls for the foregoing observations. They included convalescents from typhoid fever, relapsing fever, acute rheumatic fever, erythema multiforme and variola. All were negative.

No agglutinins were found in the serum of 3 typhus fever patients, examined 4, 5, and 6 times, respectively, during the course of the disease, and early convalescence. A similar failure to develop agglutinins

TABLE 1  
RESULTS OF AGGLUTINATION TESTS AT DIFFERENT STAGES OF THE DISEASE

Days of Disease	Number of Examinations	Number Positive	Percentage Positive
1-4	13	0	0
5-7	26	8	31
8-11	49	31	63
11-15	49	36	72
Convalescence			
1st week	53	47	88
2nd week	26	25	97
3rd week	20	18	90
After 2 mo.	13	10	77
After 3 mo.	11	6	55
After 10-14 mo.	11	3	27

in the blood is occasionally observed after other infectious diseases, such as typhoid. Persons also are encountered who, after repeated inoculations with typhoid, typhus, or cholera vaccines, do not develop agglutinins. Such an experience is not uncommon in animals used for the production of immune serums. Recovery from typhus fever and other infectious diseases is probably due to the consummation of a cellular immunity of the body, and is independent of the development of humoral antibodies. This contention receives still further support from the observation of Denzer and Olitsky,<sup>2</sup> that in contradistinction to man and monkey, the serum of guinea-pigs, after typhus fever, does not contain any agglutinins, precipitins or complement-fixing antibodies, the only immune substances demonstrable being opsonins, and

<sup>2</sup> Jour. Infect. Dis., 1917, 20, p. 99.

certain specific anaphylactic antibodies demonstrable by the Dale method.

As seen in Table 1, agglutinins are first demonstrable in the blood at about the end of the 1st week of the disease. At this time, agglutinin in more than two-thirds of the cases is still absent. During the 2nd week of the illness over two-thirds of the cases become positive. Agglutination is therefore of some diagnostic value in the latter half of the disease.

The clinical recognition of cases, as a rule, is only difficult at the beginning of the illness. From an epidemiologic standpoint, laboratory aids in diagnosis at this period of the disease are therefore of greater importance. In appreciation of this fact, some of the Austro-Hungarian field laboratories, situated in typhus-infested districts of Volhynia and Russian Poland, are equipped to examine excised specimens of skin, microscopically. But the characteristic changes described by Fraenkel only appear in the skin with the appearance of the rash on about the 5th day of the disease. Such examinations have, therefore, little advantage over the agglutination test.

In the recognition of mild, atypical, and ambulatory cases, agglutination proved to be of greater value. Such cases were frequent in Volhynia, especially among the Russian peasantry, which was undoubtedly often responsible for the spread of disease. The cases found there usually showed a high titer — a point of some importance — and the agglutinins were usually demonstrable in the serum early in the disease, by the 5th or 6th day.\*

Olitsky has noted the fact that when a blood culture made during the disease was negative, the serologic examination at that time usually showed a high content of antibodies. In my recent blood-culture studies, I have been able to confirm this. This observation together with the one mentioned would seem to indicate that the early appearance of agglutinins is an evidence of resistance to the disease, and is therefore of prognostic value. Unfortunately, however, the mortality in typhus fever is in part due to complications, chiefly pneumonia, erysipelas, and other infections.

During the early period of convalescence, a great majority of cases showed agglutinins in the blood, the maximum being reached during the 2nd week, when 97% of the examinations were positive. After this, the agglutinins slowly disappeared.

\* In a subsequent paper, the protocols of some of the mild atypical cases will be presented, also those of another group in which agglutinins appeared after exposure to infection, but without the development of any clinical evidences of the disease.



The demonstration of agglutinins in the blood during convalescence, aside from being of value for confirming the diagnosis, was sometimes epidemiologically of considerable importance. By this means we were able to obtain positive evidence of infection in persons suspected of having recently had typhus fever, but who were not seen during the illness. In view of our previous experiences, we were able, therefore, to recognize such recent cases and to recommend isolation until the homes were cleaned up and the lice which they harbored were destroyed.

TABLE 2  
RESULTS OF EXAMINATION FOR AGGLUTINATION TITERS ON ONE HUNDRED CASES  
OF TYPHUS FEVER

Day of Disease	No. of Examina- tions	Nega- tive	1:50	1:100	1:200	1:400	1:800	Average
1	3	3	0	0	0	0	0	0
2	1	1	0	0	0	0	0	0
3	5	5	0	0	0	0	0	0
4	4	4	0	0	0	0	0	0
5	5	2	1	0	0	0	0	1:17
6	15	9	6	0	0	0	0	1:20
7	8	6	1	0	1	0	0	1:30
8	12	6	6	0	0	0	0	1:25
9	12	4	7	1	0	0	0	1:38
10	12	5	5	1	0	1	0	1:62
11	15	3	7	3	0	0	0	1:54
12	17	4	7	3	3	0	0	1:74
13	9	3	3	0	2	1	0	1:103
14	23	6	6	4	2	5	0	1:135
Convalescence								
Days, 1-3	31	4	6	4	4	7	1	1:165
Days, 4-7	22	2	7	3	6	2	2	1:191
Days, 8-14	26	1	8	9	3	2	3	1:199
Days, 15-21	20	2	6	6	3	1	2	1:175
After 2 mo.	13	3	4	3	2	1	0	1:100
After 3 mo.	11	5	2	1	2	1	0	1:91
After 10-14 mo.	11	8	1	1	1	0	0	1:32

The agglutination titers of 271 examinations made on 100 cases of typhus fever have been grouped in Table 2, according to the day of the disease, or period of convalescence on which they were made. The curve (Fig. 1), which has been plotted from the average daily titers in Column 9 of this table, renders a detailed discussion of the results unnecessary. It graphically portrays the average course of the agglutinins in typhus fever.

The agglutination curves of individual cases showed a wide variation from the average. For example, in exceptional cases, a titer of

1:50 is found as early as the 5th day of the disease; in others, agglutinins are first demonstrable during the 1st or even 2nd week of convalescence. In the majority of cases, a titer of 1:50 appears on about the 10th or 11th day, or about the time when the temperature begins to fall. It then rapidly rises as the temperature approaches normal, the rise usually continuing during the early part of convalescence. The maximum titer (an average of 1:200) is reached by the end of the 1st or beginning of the 2nd week of convalescence.

After reaching this height, the titer diminishes slowly, until, in most of the cases, agglutination becomes negative (less than 1:50) after 3 or 4 months. However, in 3 cases of the series, the serum still

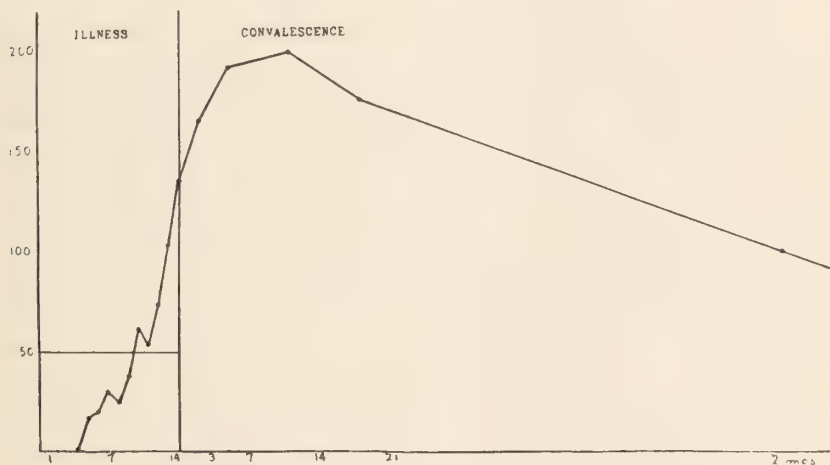


Fig. 1.—Agglutinin Curve in Typhus Fever.

contained agglutinins 12 months after the illness, the titer being 1:50, 1:100, and 1:200, respectively. In the Balkans, Plotz found an agglutination titer of 1:200 in a Bulgarian physician who had had typhus fever 21½ years previously.

The curve of agglutinins is therefore characteristically an immunity curve. It rises with the recovery and development of immunity, and then persists for a long time, gradually and slowly diminishing. This latter phase is of especial importance in view of recently reported observation of the transitory appearance in the blood during typhus fever of agglutinins for other organisms.

It has been occasionally noted that the Widal reaction may become positive during any febrile disorder in persons who previously had

typhoid fever, or who have recently been vaccinated with typhoid bacilli. This seems to be especially common during typhus fever. Many of my patients had a Widal reaction of 1:100-1:200 near the end of the disease, and in all a history of a previous typhoid vaccination, or typhoid fever could be elicited. In one Austrian soldier with typical typhus fever, who had received his last typhoid inoculation 4 weeks previously, the Widal test during the 1st week of the disease was negative. Another test made on the next to the last day of his illness showed an agglutination titer of 1:1600 (macroscopic and microscopic methods). Two weeks after the crisis it had fallen to 1:50, and another examination made just before his discharge from the hospital a week later was negative.

This temporary lighting up of the Widal reaction during typhus fever was commonly observed by the German and Austro-Hungarian army bacteriologists in Russian Poland and Volhynia, for all the troops had received typhoid vaccinations. In 1915, Weil and Felix<sup>3</sup> reported from Volhynia that the serum of persons with typhus fever or recovering therefrom agglutinated a bacillus which they isolated from the urine of 2 patients, and which subsequently proved to be a proteus bacillus. Since then other Austro-Hungarian bacteriologists have confirmed the observation, but have found also that not all proteus-strains are agglutinable by typhus serum, and that occasional strains of the colon bacillus are also agglutinated.

A systematic study with all these organisms including *B. typhi-exanthematici* has been recently made by Paneth,<sup>4</sup> director of one of the Austrian field laboratories, on about 300 cases of typhus fever. He has found that the agglutinins for the proteus bacillus and the colon bacillus, like those for the typhoid bacillus, are merely transitory. In fact, the curves for the development of agglutinins for these 3 organisms are identical with one another, but are totally different from that for *B. typhi-exanthematici*. The agglutinins of all 4 organisms usually appear in the blood during the early part of the 2nd week of the disease. The agglutinins for the typhoid bacillus, the colon bacillus, and the proteus bacillus then increase much more rapidly than do those for *B. typhi-exanthematici*, and reach their maximum at about the time when the temperature of the patient reaches normal. They then diminish rapidly, disappearing from the blood within a few weeks. The agglutination curve for *B. typhi-exanthematici*, on the other hand,

<sup>3</sup> Wien. klin. Wchnschr., 1916, 29, p. 33.

<sup>4</sup> Verhandl. d. Cong. f. inn. Med., Warsaw, 1916.

coincided exactly with the one in this paper, the average maximum concentration of agglutinins being reached during the 2nd week of convalescence, the agglutinins then persisting in the blood for many months.

The rapid appearance and disappearance of agglutinins for the proteus bacillus, the colon bacillus, and for the typhoid bacillus in persons who have received typhoid vaccine or have had typhoid fever, can only be regarded as a mobilization of preformed antibodies. Their transitory character indicates that after the termination of the typhus fever there is no continued production, as is the case with agglutinins for *B. typhi-exanthematici*. Their presence in the blood can therefore only be regarded as incidental.

These observations help to explain the fact that specific agglutinins and other antibodies have been found in typhus fever for various aerobic bacteria, previously championed as the causative agents (Müller,<sup>5</sup> Fuerth,<sup>6</sup> Rabinowitsch<sup>7</sup>). They also probably represented mobilization of antibodies performed against any members of the natural flora of the body.

The principle introduced by this observation is of considerable importance. The mere demonstration that in a febrile disease antibodies against a certain bacterium are regularly present in the blood for a brief period does not necessarily imply that that organism is the etiologic agent in the disease. As can be pointed out for many febrile diseases, in which the causative agent is known, it is the continued persistence of humoral antibodies which is usually characteristic and, therefore, of etiologic significance. As Paneth and I have shown for agglutinins, and Olitsky, for agglutinins, precipitins and complement-fixing bodies, the persistence of antibodies for *B. typhi-exanthematici* in the blood of persons for many months is, therefore, additional evidence that this organism is the causative agent in typhus exanthematicus.

<sup>5</sup> Münch. med. Wchnschr., 1913, 60, p. 1364.

<sup>6</sup> Centralbl. f. Bakteriöl., I, O., 1914, 51, p. 79.

<sup>7</sup> Centralbl. f. Bakteriöl., I, O., 1909, 52, p. 173. Berl. klin. Wchnschr., 1914, 51, p. 1458.



## THE POSSIBILITY OF TYPHOID INFECTION THROUGH VEGETABLES \*

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Vegetables, grown on land fertilized with night soil and eaten raw, have long been regarded as a possible source of typhoid infection. A few cases are on record which have been attributed to this cause.

Warry<sup>1</sup> reported in 1903 an outbreak of 110 cases occurring in Hackney, a suburb of London, considered due to the eating of watercress grown in beds fertilized with sewage. Pixley<sup>2</sup> records 2 cases of typhoid from eating uncooked rhubarb, which was grown in soil known to have been fertilized with typhoid excreta. Morse<sup>3</sup> attributed 49 cases among the inmates of an insane asylum to the eating of celery. In this instance, there had been typhoid fever in the institution some months previous and the celery beds had received the hospital sewage. The disease developed soon after the celery came into use.

An outbreak occurred in Philadelphia apparently due to contaminated watercress served at a wedding breakfast June 24, 1913, with 43 guests in attendance. Of the 19 persons who ate watercress sandwiches, 18 were ill with typhoid on July 22. Investigation by the Philadelphia Bureau of Health<sup>4</sup> showed that the watercress had been secured from a farm where sanitary conditions were quite unsatisfactory. Typhoid bacilli were not isolated from the cress beds, but all the other circumstances of the outbreak afforded strong reason for suspecting the watercress to have been the vehicle of infection.

The explanation for the few successful attempts to trace an epidemic to vegetables is apparent. Ordinarily, such articles of food are distributed among a wide circle of consumers after passing through the hands of several dealers; the difficulty of discovering weeks afterward, what vegetables were eaten and by whom, and the final tracing of them to their source of contamination may baffle the epidemiologist.

In order that vegetables shall serve as a means for disseminating typhoid from contaminated soil, there must be at least 2 positive factors.

The viability of the typhoid bacillus must extend from the time of manuring the beds until the vegetables have been consumed. In the majority of cases, the time would be approximately that required for

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<sup>1</sup> Lancet, 1903, p. 1671.

<sup>2</sup> New York Med. Jour., 98, 1913.

<sup>3</sup> Rep. Bd. Health Mass., 1899, p. 761.

<sup>4</sup> Engineering News, Aug. 14, 1913.

the growing and marketing of the vegetables, since manuring of the soil usually takes place a short time before the planting or during the growing season.

The typhoid bacillus must also adhere to the plant with sufficient tenacity to withstand the natural precipitation and other factors during the growing season, as well as those of marketing, and must survive the cleaning methods ordinarily employed in the preparing of such food for table use.

The longevity of the typhoid bacillus in soils has been a disputed question. Differences in the reports of observers can be largely accounted for by the artificial conditions used in some experiments, as well as by the faulty methods used in isolation and identification.

The results of the more trustworthy experiments indicate that the longevity of the typhoid bacillus in soil is greater than that required for the production of many common, truck vegetables.

The most definite experimental work on the infection of vegetables grown in typhoid-infected soil has been conducted by Creel,<sup>5</sup> who reports the isolation of *B. typhosus*, after a period of 25 days, from the leaves and upper stems of lettuce and radishes grown under hot-house conditions in soil contained in glass jars. From plants grown in the open, he isolated the organism in 2 experiments from the leaves and stems after 31 and 10 days, the approximate exposure to direct sunlight being 138 and 84 hours, respectively. In this work, the method used in inoculating the soil in each case was by adding a fecal emulsion mixed with 24-hour agar cultures of *B. typhosus*. To gain further information about the possibility of infection by this means, under approximately natural conditions, and also to compare such results with those under modified conditions, the following experiments have been made on vegetables grown in the hot-house and in the open.

#### INDOOR EXPERIMENTS

Preliminary experiments were conducted during the winter 1914-1915 under hot-house conditions. Radishes and lettuce were grown in soil contained in large earthen-ware, screened pots. Shortly after the planting of the seeds, inoculation of the soils was made by the addition of suspensions of *B. typhosus*. In some cases, a suspension from agar slants in physiologic salt solution was used; in others, a broth suspension. Two strains were used: One was an old stock culture, desig-

<sup>5</sup> Pub. Health Rep., 1912, 27, p. 187.

nated as Strain 1; the other, Strain 2, was isolated 21 days previously from fresh feces.

Tests were begun shortly after the appearance of the plants above the surface and repeated at intervals of 3-5 days. The lettuce stems were cut off above the surface, without allowing the leaves to come in contact with the soil; in the case of the radish the entire plant was removed from the soil and the tops cut off. The leaves and stems of the lettuce and the roots of the radishes were then placed in separate shallow pans and washed 2-3 minutes in running water, during which time they were rubbed with the hands, protected by sterile rubber gloves.

The vegetables were then removed and rinsed 2-3 times in sterile water, and finally placed in a sterile mortar and finely macerated in a few cubic centimeters of sterile water. Plates were then made from the final product and the rinse water.

#### TECHNIC

For plating, freshly prepared Endo's medium was used. I have obtained the best results in the preparation of the medium by using 7 c.c. of a saturated alcoholic solution of basic fuchsin, and decolorizing by the addition of a 10% solution of sodium sulfite, until a faint pink remained on cooling, rather than by adding a definite amount of sodium sulfite.

The plates were arranged in 6-8 Series, poured and allowed to harden with the covers partly removed; allowing 20-30 minutes for the medium to become firm. The fluid from the macerated plants and their rinse water was then transferred with sterile pipettes to the centers of the first 4-5 of the series of plates in amounts of 10, 5, 2, and 1 drops, respectively. The liquid in the center of the plates was spread over the surface with a glass rod, about 5 mm. in diameter and 18 cm. long, and bent at a point 9.5 cm. distant from 1 end so that the 2 ends of the rod were parallel and a few millimeters apart. The other end of the rod at a point immediately above the center of the 9.5 cm. length was made at a right angle and attached to a piece of thin-wall, Para rubber tubing of suitable diameter, and 3-4 cm. in length. In the other end of the rubber tubing was inserted a piece of glass rod 6-8 cm. long, which served as a handle. To obtain flexibility, a distance of 0.5-1 cm. was allowed between the ends of the glass rods inside the tubing. An ordinary T tube will serve the purpose equally well by cutting and sealing the ends of the long surface at a proper length to fit loosely inside the bottom of a Petri dish and attaching the handle and rubber to the remaining end.

In spreading a series of plates by this method, the spreader is brought in contact with the medium and fluid of the 1st plate of the series, and with the handle in a perpendicular position, is rotated through 180°. It is then transferred directly to the 2nd plate, and the process repeated, and so on through the series of plates. As the last 1-3 of the series of plates contain none of the fluid, their inoculation is made by carrying over decreasing amounts from the previous plates with the spreader. This method has an advantage over the ordinary L-rod, in that the distribution of the colonies over the surface of the plate is

more even, and because of its flexibility, the pressure is uniform on all parts of the plate and the medium is not marred.

Sterilization is best effected by autoclaving several of the spreaders in a suitable container, preferably a metal box with the top partly removed.

In each test, a control series of plates was made with a known culture of *B. typhosus*. After 18-24 hours' incubation at 37 C., the plates were examined and a number of typhoid-like colonies picked and transferred together with the typhoid control to Russell's medium. In examining the plates for typhoid colonies, I found it advantageous to compare the typhoid-like colonies with those on the typhoid-control plate, having approximately the same number of colonies as the total number on the plate under examination, since the typhoid colonies differ in appearance on heavily and lightly seeded plates.

After 24 hours' incubation at 37 C., the cultures on Russell's medium were examined. If the characteristic reaction was obtained in any of the tubes, the surface growth was washed off with sterile salt solution, the suspension filtered, and a macroscopic agglutination test made with antityphoid serum. If agglutination occurred, the culture was replated and further tested as to its action in lactose, dextrose, and dextrin broths, gelatin, neutral red, and peptone (peptone tested after 7 days for indol-production). Identification was considered complete in case the culture was agglutinated in a dilution of 1:1000 of serum, and gave characteristic reactions on the media referred to above.

In case none of the colonies picked from the Endo plates on 1st examination gave the characteristic reaction in Russell's medium, the plates were again examined and more colonies transferred. In some instances in this 2nd, or 48-hour examination of the Endo plates, *B. typhosus* was isolated after failure in the 1st 18- or 24-hour examination. Two or 3 consecutive, negative tests were made from the plants before an experiment was complete. In each test, not less than 30-50 Endo plates were made and a like number of colonies transferred to Russell's medium.

In cases where the longevity of the typhoid bacillus was determined in the soil, about 1 gm. of the soil was used and added to 1 liter of sterile water in an Erlenmeyer flask and shaken; small amounts of the mixture were then transferred with sterile pipettes to Endo plates and examination made by the same method as was used for the vegetables.

#### RESULTS OF INDOOR EXPERIMENTS

*Exper. 1.*—Lettuce was grown in sandy soil which was inoculated, before the plants appeared above the surface, with Strain 1, by adding the surface growth from 5 agar slants, after 24 hours' incubation at 37 C., suspended in one-half liter of physiologic salt solution.

TABLE 1

RESULTS OF TESTS ON LETTUCE GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS* (STRAIN 1)

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
Dec. 2	Dec. 5	Washed leaves and stems after maceration in water	Dec. 23	18
.	Dec. 5	Soil	Jan. 27	53

In 2 later tests, one Dec. 28 and the other on Jan. 4, *B. typhosus* was recovered from the rinse water.



*Exper. 2.*—Radishes were grown in garden soil to which had previously been added a small amount of fine manure. Inoculation of the soil was made with Strain 1, using 10 c.c. of a 24-hour broth culture, diluted with 500 c.c. of sterile dilute sewage.

TABLE 2

RESULTS OF TESTS ON RADISHES GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS* (STRAIN 1)

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
Dec. 2	Dec. 5	Washed roots and stems	Jan. 27	53
	Dec. 5	Soil	Feb. 17	74

Negative results were obtained in 1 examination from the washed plants on Jan. 13. On Jan. 27, the organism was not recovered from the rinse water, but was isolated from the 1st rinse water in a test on Feb. 2. In an examination from the soil on Feb. 13, negative results were obtained.

*Exper. 3.*—The soil used in this experiment was from the same source as that used in *Exper. 2*. Radish seeds were sown on April 3 without further enrichment. Inoculation was made with Strain 2, which had been isolated from a fresh typhoid stool on March 15, and had been transferred 5 times on agar. The surface growth from a one-liter flask, after 24 hours' incubation at 37 C. and suspended in 500 c.c. of tap water, was added to the surface of the soil.

TABLE 3

RESULTS OF TESTS ON RADISHES GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS* (STRAIN 2)

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
Apr. 3	Apr. 5	Washed roots and stems	May 10	35
	Apr. 5	Soil	May 24	49

In this case the 1st test could not be made until May 6. At this time, a positive result was obtained with the washed roots and stems. A 2nd test on May 8 was negative. On May 23, negative results were obtained from the soil.

#### OUTDOOR EXPERIMENTS

Experiments were begun in the open in May, 1915, and continued through the growing season. A plot of ground was selected, which afforded ideal conditions in regard to natural outdoor conditions, such as exposure to sunshine, rainfall, and atmospheric changes. Two beds, 4 by 6 feet were prepared by removing the soil to a depth of 12 inches, and filling in with garden soil, then adding a small amount of fine

manure. The soil was prepared for seeding in a manner similar to that used under ordinary conditions. The beds were screened to prevent the danger of chance spread of infection by insects. The seeds were planted in rows of ordinary width. After the plants appeared above the surface of the soil they were cultivated at intervals 7-10 days.

Typhoid stools were used for inoculation; in each case from the feces used, Endo plates were made and examined in the usual manner, and only those samples which showed the presence of *B. typhosus* in considerable numbers were used. In case the colonies picked from the Endo plates gave the characteristic reaction in Russell's medium but failed to show agglutination with antityphoid serum, the sample was discarded. In 2 samples this was found; however, in tests made after the culture had been transferred several times on agar, both showed agglutination.

*Exper. 4.*—Radish and lettuce seeds were planted in one of the beds on May 19. On the 4th day after planting, and before the vegetables were above the surface, the soil was inoculated. One c.c. of a mixture of fresh typhoid feces and urine was distributed on the surface of 3 one-liter agar flasks, and incubated for 18 hours at 37 C. The growth was washed off and added to the original stool. This mixture was then diluted in about 6 liters of water and sprinkled on the soil with an ordinary garden sprinkler, after which time the soil received no further water except by natural precipitation. The examinations were made in the manner described, with the exception that in this and succeeding experiments, an examination of the rinse water from the vegetables was not made, but larger amounts of the fluid from the macerated plants after washing was plated out.

TABLE 4

RESULTS OF TESTS ON LETTUCE AND RADISHES GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS* FOUR DAYS AFTER PLANTING

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
May 19	May 23	Washed leaves and stems of lettuce	June 13	21
May 19	May 23	Washed roots and stems of radishes	June 29	37
	May 23	Soil	July 3	41

The vegetables in each case were removed from the beds 2 days prior to testing and allowed to remain in the laboratory.

From May 23 to June 13, the respective dates of soil inoculation and removal of the lettuce previous to the last positive test, there were 138.9 hours of sunshine, a total precipitation of 3.47 in., with an average relative humidity of 73.9%. The mean temperature during the period was 57.7 F. From May 23 to June 27, the respective dates of soil inoculation and removal of the radishes previous to the last testing, there were 276.2 hours of sunshine, 6.21 in. of rainfall, a mean temperature of 60.7 F. and a mean relative humidity of 70.8%.

*Exper. 5.*—Radish seeds were planted July 5, in a bed to which fresh typhoid stools had been added 4 days previously. Approximately  $\frac{1}{2}$  liter of excreta was used, which was at once diluted with about 6 liters of water and added directly to the soil. Two days previous to each test, the radishes were removed from the soil and exposed to laboratory conditions.

TABLE 5  
RESULTS OF TESTS ON RADISHES GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS*  
FOUR DAYS PREVIOUS TO PLANTING

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
July 5	July 1	Washed roots and stems	July 29	28
	July 1	Soil	Aug. 4	34

From July 1 to July 27, there was a total of 211 hours of sunshine, 4.1 in. of rainfall, and a mean relative humidity of 66.16%. The mean temperature was 70 F. Negative results were obtained on July 23 and July 26, in examination of the radishes.

*Exper. 6.*—Radish seeds were planted on July 17 and the soil inoculated in the same manner as in *Exper. 4*.

TABLE 6  
RESULTS OF TESTS ON RADISHES GROWN IN SOIL INOCULATED WITH *B. TYPHOSUS*

Date Planted	Date Inoculated	Material Examined	Date of Last Positive Examination	Length of Time after Inoculation (days)
July 17	July 17	Washed roots and stems	Aug. 21	35
	July 17	Soil	Aug. 21*	35

\* No further tests were made on the soil after this date.

In this experiment, the vegetables to be tested were removed to the laboratory 3 days previous to the testing.

The total hours of sunshine was 206.7; precipitation, 5.65 in.; mean relative humidity, 77%; and mean temperature, 70.5 F.

In *Expers. 4, 5, and 6*, the vegetables at about 20-25 days after planting were equal in size to those ordinarily found in the markets.

The possibility of *B. typhosus* entering the interior of the plant, through injury to the roots during cultivation, suggested itself. To determine if such might occur, radishes which were about one-half matured in soil contained in pots which had not been inoculated with *B. typhosus* were used. The small root tips were injured by crushing, the tops of the pots were covered with heavy paraffined paper and sealed on the edges. The tops of the radishes were allowed to protrude through holes cut in the paper. The stems, at the point of passage through the paper were carefully wrapped with cotton. By this means no communication of the bacteria could take place from the soil to the top of the plant, except through the stems. A suspension of *B. typhosus* was then added to the soil through a glass tube, entering below the paraffined paper. The tests consisted in examining the leaves and upper stems at periods of 2-3 days for the typhoid organism, by the method used in the other experiments. In no cases were results obtained which would indicate a transference of the organism through the interior of the plant.

As a further test, radishes which had been taken from an infected bed were seared over the surfaces. They were then cut in halves, and the cut surfaces rubbed over Endo plates. The parts were then macerated in physiologic salt solution and the mixture planted. In no instance was the organism found in the interior of the plants.

Experiments were made in the autumn of 1916 for the purpose of comparing the longevity of old and freshly isolated strains of *B. typhosus* in both sandy and garden soils.

The results are shown in Table 7. Strain 3 has been used as a laboratory culture for several years. Strains 4 and 5 were isolated from the fresh stools of typhoid patients and used 2 and 5 days, respectively, after isolation.

The sandy soil was quite dry at the time of the inoculation and contained a small amount of organic matter. The garden soil was approximately the same, and prepared similar to that previously described in the outdoor experiments.

The soils were seeded Sept. 4, using for each plot (4 by 6 ft.) the 24-hour surface growth from 3 one-liter, slant agar flasks, which was diluted with 6 liters of water and sprinkled on the surface, after which the soil received no further addition of moisture except that of natural precipitation.

Tests were made by the previously described method, using a mixture of soil from the surface to a depth of 5 in.

TABLE 7

RESULTS OF TESTS COMPARING THE LONGEVITY OF OLD AND FRESH STRAINS OF *B. TYPHOSUS* IN SANDY AND GARDEN SOILS

Soil	Strain of <i>B. Typhosus</i>	Date of Last Positive Exam.	Length of Time after Inoculation (days)	Hours of Sunshine	Total Precipitation (in.)	Mean Temp. (F.)
Sandy	3	Oct. 10	36	312.8	2.15	63.1
Sandy	4	Oct. 3	29	248.6	1.77	61.5
Garden	3	Nov. 1	58	441.3	3.6	57.5
Garden	1	Oct. 24	50	384.4	2.59	58.5
Garden	4	Oct. 6	32	272.9	1.77	62.0
Garden	5	Oct. 17	43	361.8	2.58	60.4



## DISCUSSION

It is a comparatively easy matter in the beginning of such experiments here reported to recover the typhoid bacilli; but in the latter part, as the specific organisms decrease in numbers, their isolation becomes quite difficult. There is present at this time a resistant minority which persists after the greater number have disappeared. The number is small in comparison with the total bacterial flora, but it would be unreasonable to conclude that even after several negative tests, typhoid bacilli might not be present in sufficient numbers to cause infection. For this reason, too definite conclusions cannot be drawn as to the longevity of *B. typhosus* in soils. However, the marked differences in the viability which have been shown by the old and fresh cultures, particularly, in Exper. 7, indicate that long-continued growth outside the human body tends to add to their resistance in soil. The lesser differences in cases of fresh cultures also suggest considerable variation even with freshly isolated strains. This point may explain, in part, the discrepancies in the reports of many workers on the viability of *B. typhosus* in soil.

The extreme tenacity with which the typhoid organisms adhere to the surface of the plants grown in infected soils is shown in these experiments. The methods of washing the vegetables have probably been as thorough as those employed ordinarily in the preparation of such foods for table use. In but few instances has washing freed the plants of the bacilli. For this reason, vegetables from infected soil may be regarded unsafe, as long as typhoid bacilli survive in the soil.

The temperature throughout this work has shown no great variation; therefore, no conclusions have been reached relative to this factor. Determination of the longevity in frozen soils has not been undertaken.

In the outdoor tests, the conditions in Exper. 5 were identical with those which might occur naturally. Experiments 4 and 6 differed only in that they received heavier inoculations. In each of these experiments, *B. typhosus* was isolated from the plants after they had reached maturity. These vegetables had been under conditions identical, so far as possible, with those obtained in natural growing, marketing, and cleaning of such foods.

It is not only important to examine the leaves and stems in such experiments, but also to examine the root where it is used for food. The root is in contact with the infected soil and protected from sun-

shine and other conditions unfavorable to the viability of the organism and consequently is more likely to transmit infection. This has been shown in Expts. 1 and 4.

A brief attempt was made to determine the extent to which the manuring of soils with human excreta is carried on in this country. Letters of inquiry requesting such information were sent to health officers and others connected with public health work in various localities. Replies from the majority conveyed but little definite information. In 2 localities, it was found that this practice was in vogue to some extent. It is probably not so uncommon as is generally supposed, especially in suburban, village, and rural districts, where the methods of sewage disposal are more or less inadequate.

#### SUMMARY AND CONCLUSIONS

In the work herein reported, the longevity of *B. typhosus* in soils has shown considerable variation, under like conditions in the open. The old strains (1 and 3) survived in garden soil 50 and 58 days, while the viability of fresh cultures (Strains 4 and 5) was 32 and 43 days, respectively.

In sandy soil, the longevity of Strain 3 was 36 days; that of Strain 4, 29 days.

In 3 outdoor experiments, extending from May to September, *B. typhosus* was isolated from garden soil inoculated with typhoid excreta after 41, 34, and 35 days.

Under hot-house conditions in sandy soil, Strain 1 survived 53 days. The longevity was increased under the same conditions in garden soil enriched with sterile sewage and broth to 74 days. Similarly, the viability of fresh culture of Strain 2 in garden soil was 49 days.

No evidence has been found that would indicate the entrance of *B. typhosus* into the interior of the plants.

The organisms became attached to the surfaces from their contact with the soil and are not removed by ordinary washing.

Under natural conditions, radishes grown in contaminated soil were found to be still infected with typhoid bacilli in 3 experiments after periods of 37, 28, and 35 days, respectively; and from lettuce, after 21 days. This is ample time for the maturing of such vegetables.

It may be reasonably concluded that vegetables grown in soil fertilized with fresh typhoid excreta shortly before planting or during the

growing season are likely to be contaminated at the time they reach the consumer.

Vegetables so contaminated are not made safe by the ordinary methods used in the preparation of such foods for table use, and may, therefore, be a source of typhoid infection.

# THE VOGES-PROSKAUER AND CORRELATED REACTIONS OF COLI-LIKE BACTERIA \*

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It is becoming increasingly apparent that all nonsporing, lactose-fermenting bacteria are not of equal sanitary significance. There is no doubt that a water containing the relatively nonresistant *B. coli* communis and *B. communior*, or their very close allies is more objectionable and dangerous than one containing only the more ubiquitous and quite resistant *B. aerogenes* or *B. cloacae*. These 2 groups of lactose-fermenting organisms should be differentiated, and studies on rapid methods for their isolation, separation, and identification are sorely needed. It is the primary purpose of this paper to record the results of attempts to hasten the Voges-Proskauer reaction and to point out its apparent correlation with the fuchsin-aldehyd test.

## THE FUCHSIN-ALDEHYD REACTION

If an aldehyd is added to a solution of basic fuchsin, decolorized with sulphurous acid, a distinct red to violet color appears usually in less than 1 or 2 minutes. This reaction is accepted by chemists as specific for aldehyds and is universally employed as a group test. In all probability, the red coloration on Endo agar, so characteristic of *B. coli*, is merely a localized fuchsin-aldehyd test. It should be noted, however, that in the Endo medium, the fuchsin is decolorized with  $\text{Na}_2\text{SO}_3$  and not with  $\text{H}_2\text{SO}_3$ . At the suggestion of Dr. R. E. Buchanan, this reaction was tried with a number of coli-like organisms to determine if it is of any differential value. All of the organisms studied were isolated from soil. The test was made as follows: To 2 c.c. of a 72-hour culture of the organism in 0.5% carbohydrate-peptone-dipotassium-phosphate solution, were added 2 or 3 drops of basic fuchsin decolorized with sodium sulphite. A slight pink coloration was recorded as negative while a distinct red or cherry color was recorded positive. The results are shown in Table 1.

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TABLE 1  
COMPARISON OF METHYL-RED AND VOGES-PROSKAUER REACTIONS FROM GLUCOSE WITH THE  
FUCHSIN-ALDEHYD TEST

	Fuchsin-Aldehyd Test			
	Glucose		Lactose	
	+	—	+	—
Methyl-Red +	47	0	32	0
Methyl-Red —	7	115	30	25
Voges-Proskauer +	10	114	32	25
Voges-Proskauer —	44	1	30	0

\* Basic fuchsin decolorized with sodium sulphite. Figures indicate number of strains.

Of 122 organisms which were alkaline to methyl red in 0.5% glucose, only 7 (5.7%) gave a positive fuchsin-aldehyd test while all (100%) of 47 strains, which were acid to methyl red, were positive for this aldehyd test. Again, among 124 cultures which gave the Voges-Proskauer reaction, only 10 (8.0%) were positive with the fuchsin test as compared with 44 (98.0%) of 45 Voges-Proskauer negative strains.

The correlation between the methyl-red or Voges-Proskauer reaction with the modified fuchsin-aldehyd test is evident. It must not be concluded, however, that the *B. aerogenes* and *B. cloacae* groups do not form an aldehyd. In an unpublished study by George G. DeBord of this laboratory, it was observed that the fuchsin-aldehyd test is obtained only in an acid medium. He found that when basic fuchsin is decolorized with sodium sulphite and then acidified, only a faint coloration, if any, is observed. Similarly, the addition of formaldehyd to the reduced fuchsin also failed to produce any definite reaction. If, however, both an acid and formaldehyd were added, the characteristic aldehyd test was obtained.

The failure of the *B. aerogenes-cloacae* group to give a characteristic aldehyd reaction when so tested may therefore be merely another method of indicating a difference in acidity between the *aerogenes-cloacae* and the colon group. The difference observed may also throw some light on the significance of the various types of colored colonies occurring on Endo agar. All of 32 true *B.-coli* strains studied gave a distinct aldehyd test from lactose, while only 30 (54.6%) of 55 *B. aerogenes* and *B. cloacae* strains were positive. The aldehyd test from

glucose after 3 days' incubation at 37 C. is, therefore, better correlated with the Voges-Proskauer and methyl-red reactions than is the aldehyd test from lactose.

#### EFFECT OF CONCENTRATION OF CARBOHYDRATE ON VOGES-PROSKAUER AND METHYL-RED REACTIONS

A few simple experiments were made to determine the effect of concentration of sugar and period of incubation on the qualitative methyl red and acetyl-methyl-carbinol reactions; the rapidity with which the test for acetyl-methyl-carbinol may be recognized and the permanancy of the coloration.

Cultures of *B. cloacae* and *B. aerogenes*, known to give the Voges-Proskauer reaction, were studied. The organisms were inoculated from 24-hour agar slants into 100 c.c. of 0.5% peptone-dipotassium-phosphate water containing various concentrations of glucose or sucrose (0.5, 1, 2, and 5%), and incubated at 37 C. At intervals of 1, 2, 3, and 5 days, portions were removed and tested for acetyl-methyl-carbinol, and also qualitatively with methyl red.

The methyl-red test was made by adding 0.2 c.c. of the indicator to 5 c.c. of the culture. The reaction could, of course, be recognized immediately. To test for acetyl-methyl-carbinol, 2 or 3 c.c. of the culture were added to an equal volume of 10% KOH, and the mixture was allowed to stand exposed to the air. The presence of the characteristic eosin-like coloration was observed and recorded hourly for 5 hours, and again after 24, 48, and 72 hours. The results are summarized in Table 2.

*Methyl-Red Reaction.*—It is seen from Table 2 that *B. cloacae* and *B. aerogenes* were alkaline to methyl red after 24 hours' incubation, when 0.5% of the carbohydrates were employed, but were acid or in neutral tints with the higher concentrations of sugar. With 1.0% glucose, *B. cloacae* was alkaline on the 2nd day, while *B. aerogenes* was acid, but became alkaline on the 3rd day. In a general way, the time required for reversion from an acid to an alkaline reaction increases with the concentration of carbohydrates. With 5% glucose or sucrose, the medium was still acid after 1 month's incubation. The reversion with *B. cloacae* is more rapid than with *B. aerogenes*.

*The Acetyl-Methyl-Carbinol Test.*—It is customary to allow the KOH-culture mixture to stand for 24 hours before recording for the carbinol test, and some investigators do not record until after 48 hours.

This is extremely unfortunate as it results in an unnecessary loss of time. With the 2 organisms mentioned, a faint reaction could be recognized 1 hour after addition of the KOH, and after 5 hours, the coloration was usually as marked and distinct as at the expiration of 24 hours. The color was still recognizable after 2 days, but quite indistinct on the 3rd day. That the reaction may be seen in about 1 hour has been previously observed by Durham.<sup>1</sup> With concentrations of glucose above 1%, the medium is so highly colored that the pink or eosin coloration of the Voges-Proskauer reaction is obscured. As a positive test was obtained after 1 day's incubation at 37 C., the records of the Voges-Proskauer reaction are not included, for brevity and clearness, in Table 2.

TABLE 2

EFFECT OF CONCENTRATION AND PERIOD OF INCUBATION ON THE REACTION WITH METHYL RED

Concentration	B. cloacea and Glucose				B. cloacea and Sucrose				B. aerogenes and Glucose				B. aerogenes and Sucrose			
	Incubation Days				Incubation Days				Incubation Days				Incubation Days			
	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5
0.5%	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1.0%	+	—	—	—	+	—	—	—	+	+	—	—	+	+	+	—
2.0%	+	+	+	—	+	+	+	—	+	+	+	+	+	+	+	+
5.0%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\* Reactions in the neutral tints included as positive.

It appeared from the preliminary experiment that, if necessary, about 5 hours after the addition of KOH would be a safe time to record for the carbinol reaction as a presumptive test. This was verified by a confirmatory study of 152 strains employing 10 test substances. The results after 5 and 24 hours are indicated in Table 3.

Of 140 strains which gave the Voges-Proskauer reaction from glucose, 130 (92.9%) were positive after 5 hours. A similar result was observed with sucrose, where of 134 positive carbinol tests, 127 (94.8%) were obtained in 5 hours. With other test substances, the number and percentage of positive tests, after 5 hours, were as follows: raffinose, 114 (100%); mannitol, 116 (88.5%); salicin, 86 (82.7%); dulcitol, 11 (78.6%). With galactose and lactose, the 5-hour period was not reliable. These media became very dark on the addition of a strong alkali, thus obscuring the reaction. It is concluded from these

TABLE 3  
COMPARISON OF FIVE HOUR AND TWENTY-FOUR HOUR RECORDS OF TESTS FOR  
ACETYL-METHYL-CARBINOL

Test Substances	Total Positive Reactions	Positive in 5 Hr.		Positive in 24 Hr. Negative in 5 Hr.	
		No.	%	No.	%
Glucose.....	140	130	92.9	10	7.1
Galactose.....	96	53	55.2	43	44.8
Lactose.....	90	0	0.0	90	100.0
Sucrose.....	124	127	94.8	7	5.2
Raffinose.....	114	114	100.0	0	0.0
Mannitol.....	131	116	88.5	15	11.5
Salicin.....	104	86	82.7	18	12.3
Dulcitol.....	14	11	78.6	3	21.4
Dextrin.....	12	12	100.0	0	0.0
Starch.....	18	17	94.0	1	0.0

results that the 5-hour period may be employed as a presumptive test for acetyl-methyl-carbinol.

*On Accelerating the Carbinol Test.*—Hardin ascribes the Voges-Proskauer reaction to the production of acetyl-methyl-carbinol,  $\text{CH}_3\text{CHOH.CO.CH}_3$ , which is an oxidation product of 2:3 butyleneglycol ( $\text{CH}_3\text{CHOH.CHOH.CH}_3$ ). The carbinol becomes further oxidized to diacetyl ( $\text{CH}_3\text{CO}$ )<sub>2</sub> on exposure to air, which in the presence of strong KOH reacts with peptone to form an eosin-like compound. The problem of hastening this reaction resolves itself, therefore, into one of finding an oxidizing agent capable of oxidizing acetyl-methyl-carbinol to diacetyl, but which will not form the carbinol from butyleneglycol.

A number of experiments were tried with a culture of *B. aerogenes* and *B. cloacae*, organisms known to form acetyl-methyl-carbinol, and *B. communior*, which does not give the carbinol test as a check. The oxidizers employed were aerated potassium hydroxid, potassium dichromate, potassium chlorate, potassium perchlorate, barium peroxid, calcium hypochlorite, and hydrogen peroxid. As the reaction is more intense and would be more easily observed in sucrose media, which is colorless, this medium was employed in place of glucose.

The 1st test was an attempt to hasten the reaction by using a 10% KOH solution, through which air had been drawn over night. The



results with this aerated KOH solution were somewhat better than when plain KOH was used, but not markedly so. We then tried mixing the KOH and culture media and heating for 1 or 2 minutes, but apparently with but slight increase in speed of reaction.

The addition of an oxidizing agent was next tried. Varying dilutions of  $K_2Cr_2O_7$  ( $0.005 = 5.0\%$ ) were made in 10% KOH solution. Each concentration of dichromate was tested as follows: Five c.c. were placed in each of 3 test tubes; to 1 was added an equal volume of the culture under examination and the mixture was allowed to stand at room temperature; to the 2nd, an equal volume of the culture was added and the mixture heated for 2 minutes in a boiling water bath, after which it was placed by the side of the 1st tube. To the 3rd tube

TABLE 4  
EFFECT OF POTASSIUM DICHROMATE ON TEST FOR ACETYL-METHYL-CARBINOL

Concentration $K_2Cr_2O_7$ in %	B. communior			B. cloacae					
	3 Hours			15 Minutes			3 Hours		
	Ordinary Test	Heated	Ring Test	Ordinary Test	Heated	Ring Test	Ordinary Test	Heated	Ring Test
0.005	—	—	—	f	m	—	s	s	—
0.01	—	—	—	f	m	—	s	s	f
0.05	—	—	—	f	m	—	s	s	m
0.1	—	—	—	f	m	—	s	s	m
0.2	—	—	—	f	s	—	s	s	m
0.5	—	—	—	f	s	—	s	s	s
1.0	—	—	—	f	s	f	s	s	s
5.0	—	—	—	f	s	f	vs	vs	vs

f = faint; m = moderate; s = strong; vs = very strong.

the culture was added in such a way as to form a layer over the dichromate KOH mixture, thus permitting the formation of a ring test. The tubes were examined at the end of 15 minutes and again after 3 hours. The results are shown in Table 4. A distinct reddening, as compared with a check tube, was recorded as positive.

As  $K_2Cr_2O_7$  imparts a yellow or orange color to the solution, particularly in its higher concentrations, a weak reaction might be masked; consequently, it is not the ideal oxidizing agent for our purpose. Nevertheless, if checks are run for comparison, this reagent may be safely employed after a little practice.

It is seen from Table 4 that a concentration 0.2% or more of potassium dichromate, in 10% KOH solution, when added to a culture and the mixture heated, will accelerate the carbinol test so that a strong reaction may be observed in 15 minutes. The ring test was not found suitable with this reagent.

Among other substances tested were bleaching powder, potassium chlorate, potassium perchlorate, barium peroxid, and hydrogen peroxid. All were capable of accelerating the reaction, but the most consistent and the best results were obtained with hydrogen peroxid.

The first attempts with hydrogen peroxid were very disappointing. When employed in a manner analogous to that in which the potassium bichromate was used, no test could be obtained at all, because of the bleaching action of peroxid. After many futile attempts it was suggested that the hydrogen peroxid be added after the KOH-culture mixture was heated. This procedure was very successful. If 3 c.c. of a 48-hour sucrose-peptone culture is added to an equal volume of 10% KOH, the mixture heated in a boiling water bath for 2 minutes, then 2 or 3 drops of hydrogen peroxid added, the pink coloration of the carbinol test appears in 1 or 2 minutes and will persist for several hours. If an excess of hydrogen peroxid is used, the color will disappear in a very few minutes, or even instantly, if the excess is very great.

This rapid method of testing for acetyl-methyl-carbinol was then tried with more than 100 strains, including *B. aerogenes*, *B. cloacae*, *B. coli communis*, *B. communior*, etc., and also employing different carbohydrates. With sucrose, mannitol, raffinose, and salicin, substances giving colorless media, the hydrogen-peroxid test as applied above gave as good results as were obtained by allowing the KOH-culture mixture to stand at room temperature for 24 hours. With glucose, however, the use of hydrogen peroxid did not give as good results. The difficulty is probably due to the coloration which develops when the glucose-KOH mixture is heated.

#### SUMMARY AND CONCLUSIONS

The Voges-Proskauer reaction may be recorded, as a presumptive test, 5 hours after the addition of KOH to the test culture.

The Voges-Proskauer and methyl-red reactions seem to be well correlated with the fuchsin-aldehyd test from glucose. On addition of 2 or 3 drops of basic fuchsin, decolorized with sodium sulphite, to

2 c.c. of a 72-hour culture, the Voges-Proskauer negative and methyl-red positive strains gave a very distinct red or violet coloration, while the methyl-red negative and Voges-Proskauer positive group were pink or colorless.

The test for acetyl-methyl-carbinol from sucrose, raffinose, mannitol, and salicin may be accelerated by suitable oxidizing agents, among which the most desirable are hydrogen peroxid, barium peroxid, and potassium dichromate, but such oxidation does not give as good results with galactose or glucose.

If hydrogen peroxid is used, the mixture of the culture and potassium hydroxid should be brought to a boil over the flame or heated in a boiling-water bath for 2 minutes before addition of the oxidizing agent. The addition of an excess of the peroxid produces a transitory color due to its strong bleaching action. About 3 or 4 drops of hydrogen peroxid to 6 c.c. of the KOH-culture mixture gives good results. The pink or eosin-like coloration may be observed in 1 or 2 minutes.

# INFLUENCE OF OXYGEN TENSION ON MORPHOLOGIC VARIATIONS IN *B. DIPHTHERIAE* \*

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Denny<sup>1</sup> showed by repeated and frequent examinations of pure cultures of the diphtheria bacillus, grown on Loeffler's blood serum at 36 C., that the young forms, C<sup>2</sup> and D<sup>2</sup>, Wesbrook<sup>2</sup> types, gradually elongate, stain more and more irregularly, and tend to become club-shaped, or rarely branched, with age; further, that the rate at which these morphologic changes take place is delayed by a temperature below the optimum (19-21 C.), or, as Neisser also pointed out, the normal evolution may be suppressed by a temperature above the optimum (40 C.).

I was not acquainted with Denny's work when the following observations were made, so that they not only call attention to another physical factor in this variation, but also perhaps have some additional value in that they were not influenced by his work.

The culture used in this study was isolated from the throat of a typical case of diphtheria, on a slant of Loeffler's blood serum, kept aerobically at 37 C. The single colony subculture, after 24 hours' growth at 37 C. on the same medium, showed chiefly the A<sup>1</sup> type, along with C<sup>1</sup>, and D<sup>1</sup>, and intermediate types. The culture was plated out from salt solution emulsions by the successive stroke method, subcultures being made from well isolated colonies, 3 times. The final aerobic subculture on which the following observations were made still showed the barred types referred to above (Fig. 1).

The pathogenicity and ability to ferment carbohydrates was not tested until 9 months after isolation. For 5 months of the 9, the culture was kept at 5 C. on +1 human blood agar (anaerobic). When injected subcutaneously into guinea-pigs (0.5 c.c. of a 48-hour broth culture per 100 gm.), death occurred in 3-4 days with a typical serogelatinous edema. An anaerobic and an aerobic subculture on +0.5 Martin's medium containing human blood was tested: Slants of +0.5 Martin's medium (muscle sugar free) containing sterile ascites fluid

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<sup>1</sup> Jour. Med. Research, 1903, 9, p. 117.

<sup>2</sup> Tr. Assn. Am. Phys., 1900, 15, p. 198.



and sterile solutions of the following substances were inoculated, one set, from the aerobic that was kept under aerobic conditions; the other set, from the anaerobic which was kept under anaerobic conditions. The aerobic set showed no acid-production, but alkali-production, after 4 days at 37 C., in dextrose, levulose, galactose, lactose, saccharose, maltose, mannite, dextrin and starch, while the anaerobic culture showed marked acid-production from dextrose, only.

In the microscopic study of types, the smears were fixed by heat, after thorough drying high above the Bunsen flame, and stained for about 3 minutes with Loeffler's methylene blue.

When grown on parallel aerobic and anaerobic cultures on Loeffler's serum, the aerobic gave the foregoing types, whereas the anaerobic yielded almost entirely the D<sup>2</sup> type. Cross transfers were then made, aerobic from anaerobic and vice versa, on Loeffler's serum, and on + 0.5 and + 2.0 Martin's medium,<sup>3</sup> containing sterile human pleuritic fluid. The same inhibition in the evolution of the aerobic types was shown by all the anaerobic cultures made from the aerobic, while the aerobic cultures made from the anaerobic developed the D<sup>1</sup>, C<sup>1</sup>, and A<sup>1</sup> types.

If we determine the age of an organism, not by time alone, but by the time elapsing between youth, old age and death, then the lettering on Wesbrook's types should be reversed. The D<sup>2</sup> types are undoubtedly the youngest types, and if we can keep them in a state of suspended animation, they are still young even months later, compared with A<sup>1</sup> types, which have died in the interval. This was clearly shown by + 1 human blood-agar cultures kept for 5 months at 5 C. The 5-months-old anaerobic culture still showed the D<sup>2</sup> types (Fig. 2); these stained well and yielded luxuriant growth when transplanted aerobically and anaerobically, while the 5-months-old aerobic culture was dead.

When the D<sup>2</sup> types on an anaerobic + 1 human blood-agar culture were transferred to the same medium and incubated aerobically for 24 hours, the larger barred types developed, as shown in Figure 3.

In an anaerobic culture on Loeffler's blood serum, the growth was more luxuriant than in an anaerobic culture on + 1 human blood agar, where it was very delicate and the growth continued on Loeffler's serum at 37 C. for more than 24 hours; that is, a culture which in 24 hours showed chiefly the D<sup>2</sup> type, in 48 hours showed chiefly D<sup>1</sup> with C<sup>1</sup>, and a few A<sup>1</sup> types.

<sup>3</sup> Jour. Path. and Bacteriol., 1911, 15, p. 76.

The influence of oxygen on the evolution of the older types was shown in another way: The types present in 24-hour-old cultures on +1 human blood agar were grown at the ordinary tension, at partial tension,<sup>4</sup> with the aid of *B. subtilis*, and anaerobically were recorded. The results of the aerobic and anaerobic growths corresponded with what has already been stated. In the partial tension culture inoculated



Fig. 1.—Loeffler's serum, aerobic, 24 hours, 37 C. A<sup>1</sup>, C<sup>1</sup>, D<sup>1</sup> types, 4-6 microns  $\times$  0.8-0.5 micron; small Pyriform, about 0.9 micron by 0.5 micron (scale 1 micron = 0.2 inch).

Fig. 2.—+ 1 human blood agar slant, anaerobic, 24 hours, 37 C. D<sup>2</sup> and smaller types, 0.5-2.0 micron by 0.5-0.3 micron (scale 1 micron = 0.2 inch).

Fig. 3.—+ 1 human blood agar (inoculated with D<sup>2</sup> type from anaerobic culture), aerobic, 24 hours, 37 C; 1-6 microns by 0.8-1 micron (scale 1 micron = 0.2 inch).

Fig. 4.—+ 0.5 Martin's ascites agar, partial oxygen tension culture from subcutis of guinea-pig, 24 hours, 37 C. Longest forms 5-6 microns by 0.8 micron (scale 1 micron = 0.2 inch).

Fig. 5.—Culture similar to that shown in Fig. 4, but grown aerobically for 24 hours, 37 C, chiefly C<sup>2</sup> type. Longest forms 2-3 microns by 0.6 micron (scale 1 micron = 0.2 inch).

from an aerobic slant, there was not much delay in the evolution of types, but in the partial tension culture inoculated from an anaerobic culture (D<sup>2</sup> types), very few of the organisms had gone beyond the D<sup>1</sup> type.

<sup>4</sup> Wherry and Oliver: Jour. Infect. Dis., 1916, 19, p. 288.

As far as my work goes, it is evident that the oxygen supply has a more potent influence on the evolution of the larger types (involution forms, as some think) than has the composition of the medium, which however may influence the rate of multiplication as well as oxygen supply and temperature; that is, when grown on +0.5 Martin's medium containing human blood, this culture showed equally luxuriant growth under aerobic and anaerobic conditions; but while the aerobic in 24 hours showed chiefly A<sup>1</sup>, C<sup>1</sup>, and D<sup>1</sup> types, the anaerobic showed D<sup>2</sup>, E<sup>2</sup>, and F<sup>2</sup> types.

I<sup>5</sup> have found that many of the parasitic bacteria become adapted to a lower oxygen tension than the atmospheric. Two attempts failed to demonstrate that this held for the diphtheria bacillus in cultures on Loeffler's serum from the throats of cases of diphtheria. The growth was equally good. Perhaps this was due to the transfer of superficial aerobic organisms. However, interesting results were obtained in cultures from the subcutis of a guinea-pig which died 48 hours after inoculation. A loopful of the serogelatinous exudate was rubbed over the surface of slants of +0.5 Martin's medium, containing ascites fluid. These were incubated for 24 hours at 37 C., aerobically, and at partial tension. The partial-tension cultures showed much more luxuriant growth than the aerobic, and while in the partial-tension culture A<sup>1</sup>, C<sup>1</sup>, and D<sup>1</sup> types predominated (Fig. 4), in the aerobic culture, they were chiefly of the C<sup>2</sup> type (Fig. 5), that is, bringing the bacteria out of the tissues at partial tension enabled them to grow faster and they went on to the formation of barred types, whereas aerobic cultivation yielded chiefly the small, solid-staining type.

It is evident that in trying to interpret the evolution of the various morphologic types encountered in cultures from diphtheritic throats on Loeffler's serum, one must consider not only the variations in oxygen supply produced by associated organisms of various kinds, but also the possible influence of metabolic products set free in such mixed cultures. Denny has shown that granular forms (C, D) tend to appear in cultures mixed with staphylococci, or streptococci. It seems possible that the production of the granular types A, C, and D, may depend on the presence of certain organic acids as in the production of the granular types of tubercle bacilli.<sup>6</sup>

Wesbrook, Wilson, and McDaniel<sup>2</sup> clearly recognized and described the extreme variability of these so-called types, and never meant that they should be anything more than an aid to rapid diagnosis.

<sup>5</sup> Wherry and Oliver: Jour. Infect. Dis., 1916, 19, p. 288; 1917, 20, p. 1.

<sup>6</sup> Wherry: Centralbl. f. Bacteriol., I, O., 1913, 70, p. 115.

## . CONCLUSIONS

The observations recorded appear to show that the occurrence of various morphologic types of the diphtheria bacillus in a mixed culture from the lesions of diphtheria is to be explained only when one appreciates the influence of oxygen on the rate of growth. They confirm the work of Denny in so far as they show that the normal evolution of the barred type begins with the D<sup>2</sup> type (or smaller), and develops successively the D<sup>1</sup>, C<sup>1</sup>, A<sup>1</sup>, and sometimes the A type.

That the oxygen supply is not the only factor concerned is shown by the difference in the rate of growth and morphology in cultures grown anaerobically on media of different composition.

In mixed cultures from the throat, the morphology of diphtheria bacilli growing in association with other micro-organisms is probably modified by chemical products of growth, as well as by the reduced oxygen tension resulting from such association. Further, in making cultures from a diphtheritic membrane, one may transfer bacilli adapted to varying oxygen tensions; that is, if a bacillus adapted to a lower tension than the atmospheric is placed on the medium and grows unaccompanied by other species, its growth will be retarded and consequently its descendants will be of the solid-staining types.



# NINHYDRIN REACTION IN THE EXAMINATION OF CEREBROSPINAL FLUID \*

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In 1915 Noble<sup>1</sup> described the ninhydrin reaction, a chemical test which he used in the examination of spinal fluids. He asserted it was of value in differentiating tuberculous meningitis from other diseases in which a clear fluid was yielded.

It is well known that in tuberculous meningitis and in poliomyelitis the cerebrospinal fluid is almost invariably clear except for a slight

TABLE 1  
EXAMINATION OF 20 CASES OF TUBERCULOUS MENINGITIS; FLUIDS CLEAR

Case	Albumin	Globulin	Ninhydrin	Remarks
1	++++	++++	++++	
2	++I	++I	++	Bacilli in smear
3	++++	++++	++++	
4	++++	++++	++++	Bacilli in smear
5	++	++	++I	
6	+++	+++	++	
7	++++	++++	++++	Bacilli in smear
8	+++	+++	+++	
9	+++	+++	+++	Bacilli in smear
10	+++	+++	++	Bacilli in smear
11	++	++	++	
12	++	++	++	
13	+++	+++	++	
14	+++	+++	++	
15	++++	++++	++++	
16	+++	+++	++	
17	+++	++I	++I	
18	+++	+++	+++	
19	++++	++++	++++	
20	++++	++++	+++	

haziness as seen by transmitted light, a condition frequently found. The cytology is similar, presenting a predominance of mononuclear cells ranging from 50 to nearly 100%. The albumin and globulin content is generally higher in tuberculous meningitis and lower in poliomyelitis. Deviations are met with, and, therefore, differentiation by examination of spinal fluid is not easy. In doubtful cases, the tubercle bacilli may be demonstrated in the smear or by animal inoculation, the results of which cannot be known for 4 weeks.

\* Received for publication January 18, 1917.

<sup>1</sup> München. med. Wchnschr., 1915, 62, pp. 975 and 1786.

The ninhydrin test is simple to perform, much easier indeed than the Noguchi-globulin test. Of a watery solution of 1% ninhydrin, 0.1 c.c. is added to 0.5 c.c. of spinal fluid in a test tube, and is then shaken and boiled for a few seconds. On cooling, a purple color turn-

TABLE 2  
EXAMINATION OF 15 CASES OF MENINGISM; FLUIDS CLEAR

Case	Albumin	Globulin	Ninhydrin	Remarks
1	+	+	+	Pneumonia
2	+	+	+	Diphtheria
3	—	+	+	Pneumonia
4	+	+	+	Pneumonia
5	—	—	—	Adenitis
6	+	+	+	Peritonitis
7	+	+	+	Gastro-enteritis
8	+	+	+	Endocarditis
9	+	+	+	Pneumonia
10	+	+	+	Mastoid
11	+	+	+	Pneumonia
12	+	—	+	Pneumonia
13	+	+	+	Pneumonia
14	+	+	+	Pneumonia
15	+	+	+	Pneumonia

TABLE 3  
EXAMINATION OF 35 CASES OF POLIOMYELITIS; FLUIDS CLEAR

Case	Albumin	Globulin	Ninhydrin	Remarks
1	+	+	+	Fluid cloudy Fluid cloudy
2	+I	+I	+I	
3	+I	+I	+	
4	+I	+I	+	
5	++++	++++	+++	
6	++++	++++	++	
7	+	+	+	
8	+I	+I	+	
9	+	+	+	
10	+I	+	+	
11	+I	+I	+	
12	++	++	+	
13	+	+	+	
14	++	+I	+	
15	+	++	++	
16	+	+	+I	
17	+I	++	+++	
18	++++	++++	++++	
19	++	++	+	
20	+	+	+	
21	+++	+I	+	
22	++	++	++	
23	++	+I	+	
24	++	++	++	
25	+I	+I	+	
26	+I	+I	I	
27	+++	+++	++	
28	+	+	+	
29	++	++	++	
30	++	++	+	
31	+I	++	+	
32	+	+	+	
33	+	+	+	
34	+	+	+	
35	++	++	++	

ing into a deep blue appears. This indicates a positive reaction. A negative reaction gives either a straw-yellow color or no color at all. Intermediate shades of a plum color are noted, indicating a weakly positive reaction. Translated in terms of + signs, which are used in the examination of albumin and globulin, the colors of the ninhydrin reaction would correspond thus: Deep-blue = + + + +; straw-yellow or colorless = —; plum-color = + I or + +.

Tables 1-4 give the results of examinations of the spinal fluids in 20 cases of tuberculous meningitis, 15 cases of meningism, 29 cases of nontuberculous meningitis, and 35 cases of epidemic poliomyelitis.

TABLE 4  
EXAMINATION OF 29 CASES OF NONTUBERCULOUS MENINGITIS; FLUIDS TURBID

Case	Albumin	Globulin	Ninhydrin	Remarks
1	++++	++++	++++	Epidemic meningitis
2	++	++I	++	Epidemic meningitis
3	++++	++++	++	Epidemic meningitis
4	++++	++	++	Epidemic meningitis
5	++++	++	++	Epidemic meningitis
6	++++	++	++	Epidemic meningitis
7	++	++	++	Epidemic meningitis
8	++	++	++	Epidemic meningitis
9	+++	+++	+++	Epidemic meningitis
10	++++	++++	++++	Epidemic meningitis
11	+++	+++	+++	Influenzal meningitis
12	++++	++++	++++	Influenzal meningitis
13	++++	++++	++++	Staphylococcus meningitis
14	++++	++++	++++	Pneumococcus meningitis
15	++	++	++	Epidemic meningitis
16	++	++	++	Epidemic meningitis
17	++	++	++	Epidemic meningitis
18	++++	++++	++++	Pneumococcus meningitis
19	++++	++++	++++	Pneumococcus meningitis
20	++++	++++	++++	Epidemic meningitis
21	++++	++++	++++	Epidemic meningitis
22	++++	++++	++++	Epidemic meningitis
23	++	++	++	Epidemic meningitis
24	+++	+++	++++	Epidemic meningitis
25	++++	++++	++++	Epidemic meningitis
26	++++	++++	++++	Epidemic meningitis
27	++++	++++	++++	Epidemic meningitis
28	++	+++	++	Epidemic meningitis
29	++++	++++	++++	Pneumococcus meningitis

A study of the tables show that a distinct relationship exists between the albumin and globulin content and the ninhydrin reaction in any given fluid. There are 20 cases of tuberculous meningitis, in 5 of which tubercle bacilli were demonstrated in the smear. The ninhydrin reaction showed no greater intensity of color than the albumin and globulin would indicate. In the examination of the cerebrospinal fluids in 15 cases of meningism, in which there were only traces of albumin and globulin, the ninhydrin gave a corresponding faint reaction. In the 35 cases of poliomyelitis, the albumin and globulin con-

tent in the fluids ran, in general, parallel with the ninhydrin reaction. The relationship between the quantity of albumin and globulin and the intensity of the ninhydrin reaction was shown again by the results obtained in the examination of the fluids in 29 cases of meningitis. In these fluids also the globulin content ran generally parallel with the ninhydrin reaction.

#### CONCLUSIONS

The ninhydrin reaction in the examination of spinal fluids is of moderate differential diagnostic value.

It runs, as nearly as may be marked, parallel with the albumin and globulin content of a given fluid.

It may be used as a bedside presumptive test, but has no advantage over the albumin and globulin test, and is incomplete without the cytologic and bacteriologic examination, as it does not separate the nonpurulent pathologic fluids.



# NATURE OF PLAGUE PROTEOTOXINS\*

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In an earlier communication,<sup>1</sup> it was shown that shock-producing poisons could not be obtained with *B. pestis* when the organisms were unsensitized, however the proportions of bacteria and complement and the time element were varied. The experiments here reported are an extension of the previous studies and take up the phases of the problem of plague immunity that concern the production of powerful poisons by means of sensitized plague bacteria and the thermostability of plague proteotoxins.

*Exper. 1.*—One agar slant of *B. pestis* (24 hours) was taken up with 0.5 c.c. of NaCl solution and incubated at 37 C. for 1 hour with 0.7 c.c. of inactivated serum from Rabbit X which had been injected 5 times intravenously with *B. pestis* heated to 60 C. for 30 minutes. This animal received doses of 0.1, 0.2, 0.3, 0.5, and 1.0 agar slant and was bled 10 days after the last injection. The serum-bacteria mixture was centrifugated, the organisms washed once with NaCl solution and then incubated with 10 c.c. of fresh guinea-pig serum for 14½ hours at 37 C. After 1 hour's centrifugation, the supernatant fluid was injected intravenously into guinea-pigs.

TABLE 1  
RESULTS OF EXPERIMENT 1

Guinea-Pig	Weight gm.	Dose c.c.	Result
1	180	1.0	No shock; died of acute plague after 72 hr.
2	266	1.5	Very slight shock? Died of acute plague after 50 hr.
3	260	4.0	Slight shock? Died of acute plague after 48 hr.

In this experiment the results were not clear-cut and it seemed as if deficient sensitization was responsible for the failure to elicit typical shock. This supposition is in all likelihood borne out by the next experiment. Here, as in the work reported in a previous paper, the few organisms remaining in the supernatant fluid were able to cause death.

\* Received for publication January 18, 1917.

<sup>1</sup> Jour. Infect. Dis., 1917, 20, p. 180.

*Exper. 2.*—The method of procedure was the same as that followed in *Exper. 1*, with the exception that 2.0 c.c. of the sensitizer were used for 1½ agar slants of a Dairen strain of *B. pestis* and 1.9 c.c. of the same sensitizer for 1 slant of a Shanghai strain. The amount of complement used for each culture was 7.0 c.c. and the incubation period was 14½ hours.

TABLE 2  
RESULTS OF EXPERIMENT 2

Guinea-Pig	Weight gm.	Dose c.c.	Result
1	172	3.5	Severe shock; typical death in 5 min.
2	150	2.5	Severe shock; died within 20 hr.
3	144	3.25	Slight shock; died within 20 hr.
4	148	3.0	Moderate shock; died within 20 hr.

Definite shock could be produced with *B. pestis*, with the sensitizer used, when the organisms were homologous as well as heterologous. In the case of Guinea-pigs 3 and 4, the Shanghai strain was sensitized with its homologous serum, and in 1 and 2, a Dairen strain was used with the rabbit serum which was obtained originally by injections of a Shanghai culture.

*Exper. 3.*—In this experiment the proteotoxins were heated for 30 minutes at 50 C.

Three agar slants of *B. pestis* were taken up with 0.8 c.c. NaCl solution and incubated with 3.5 c.c. of inactivated serum from Rabbit X for 1 hour at 37 C. Fifteen c.c. of guinea-pig serum were incubated with the washed, sensitized bacteria for 14 hours. Injections were made intravenously with the supernatant fluid obtained after 1 hour's centrifugation.

TABLE 3  
RESULTS OF EXPERIMENT 3

Guinea-Pig	Weight gm.	Dose c.c.	Result
1	168	4.0	Severe shock; died in 6 hr.
2	150	3.5	Severe shock; died of acute plague after 48 hr.
3	142	3.0	Severe shock; died in 18 hr.
4	151	3.5	Severe shock; died in 21 hr.

Heating at 50 C. for one-half hour does not destroy the shock-producing properties of proteotoxin obtained with *B. pestis*. Guinea-pigs 1 and 2 served as controls, unheated material being used for injection.

*Exper. 4.*—In this and the following experiments, rabbits were substituted for guinea-pigs and horse serum was used instead of guinea-pig serum for the production of proteotoxins.

One and one-half agar slants of *B. pestis* were sensitized at 37 C. for 1 hour with 1.8 c.c. inactivated serum from Rabbit X. Ten c.c. of normal horse serum were incubated for 14 hours in contact with the washed, sensitized bacteria. After centrifugation, the supernatant fluid was heated for 35 minutes at 50-51 C. and 5 c.c. were injected intravenously in a rabbit weighing 567 grams. The rabbit had a severe shock and fell on its side at once. Dyspnea, twitching and pawing occurred. It recovered in 1 minute. The next day the animal died. Three agar slants of *B. pestis* were incubated for 1 hour at 29 C. with 4.0 c.c. of inactivated serum from Rabbit X. Twenty c.c. of normal horse serum were incubated with the sensitized organisms for 16 hours at 26-29 C. The supernatant fluid was heated for 30 minutes at 51-52 C.

TABLE 4  
RESULTS OF EXPERIMENT 4

Rabbit	Weight gm.	Dose c.c.	Result
2	412	2.5	Moderate shock; fell to side; dyspnea, pawing and trembling; recovered in 1 min. Slight shock, recovered in 1 min.
3	297	3.0	

One and one-half agar slants *B. pestis* were used with 2.0 c.c. of the sensitizer from Rabbit X and incubated for 1 hour at 30 C. Ten c.c. of normal horse serum were incubated for 14 hours at 30-31 C. The proteotoxin was heated for 30 minutes at 60-61 C. and 2 c.c. were injected into a rabbit weighing 345 gm. Severe shock resulted. It fell on its side at once. Dyspnea and continuous pawing occurred. The animal recovered in 2 minutes.

The preceding experiments demonstrate clearly that the proteotoxins obtained by the method given are thermostabile. Heating for 30-35 minutes at temperatures ranging from 50-61 C. does not destroy the toxicity or shock-producing properties.

There is a wide range of combinations within which the poisons may be obtained, as is evident from the various conditions of temperature and incubation.

*Exper. 5.*—In this experiment, heavier animals were used and the results of the previous trials were confirmed. Four slants of *B. pestis* were emulsified in a total volume of 1.0 c.c. NaCl solution and incubated with 5.5 c.c. of serum (inactivated) from Rabbit X, for 1 hour at 29 C. Thirty c.c. of normal horse serum were used for the complex. The incubation period was 14 hours at 29 C. The proteotoxin was divided into 2 lots and heated at 50 C. for 35 minutes and at 57 C. for 30 minutes, respectively. The injections, as before, were made into the marginal ear vein.

The potency of the poison is well shown in this experiment, in which an effort was made to administer doses sufficiently great to induce shock, yet enabling the animals to survive. Unless the dose given was well below that required for visible shock, according to the

weight of the animal, the outcome was invariably fatal. Death seemed to be due to a slow poisoning. This has been observed in numerous other instances which have been omitted in this report for the sake of brevity.

TABLE 5  
RESULTS OF EXPERIMENT 5; INJECTION OF PROTEOTOXIN HEATED AT 57 C

Rabbit	Weight gm.	Dose c.c.	Result
1	726	2.4	No shock.
2	776	3.2	Moderate shock; on side immediately; intermittent pawing and twitching; recovery in 2 min.; died in 20 hr.
3	587	4.0	Very severe shock; dyspnea, continuous pawing and trembling; recovered in 7 min.; died after 6 days with severe emaciation.

An analysis of these experiments indicates that the toxemic manifestations of plague bacteria are of a different nature from those which have been described for other organisms such as the typhoid bacillus, for example. The most striking feature of *B. pestis* from the standpoint of poison-production is the strict requirement of sensitization. In the case of other bacteria studied, this has never been encountered, so far as I know. Sensitization has usually hastened the formation of proteotoxins, but has not been an indispensable factor in the process, since unsensitized bacteria gave rise to powerful poisons equally well. The significance of this point does not immediately become apparent, but suggests possible explanations for the mechanism of plague infection, the relationship existing between proteotoxins and bacterial virulence and the possible importance of an antigen-sensitizer complex in the production of shock.

TABLE 6  
RESULTS OF EXPERIMENT 5: INJECTION OF PROTEOTOXIN HEATED AT 50 C.

Rabbit	Weight gm.	Dose c.c.	Result
1	973	2.0	No shock
2	883	3.6	Very slight shock; died after 6 days
3	680	2.5	Slight shock; died after 6 hr.

Much evidence has accumulated to support the belief that the different heat-stable antibodies are fundamentally alike. From the results recorded in the preceding experiments, it seems as if we might safely identify such heat-stable poisons with one of these antibodies. Zinsser<sup>2</sup> has shown conclusively that after stated intervals, resistance

may be developed to shock, subsequent to a single injection of proteotoxins obtained with typhoid bacilli. If thermostability can be proved for these proteotoxins, it will mean the formulation of a general principle.

The rôle played by the sensitizer in the mechanism of shock when *B. pestis* is used suggests the likelihood of an antigen-sensitizer complex as the important factor in plague infection. This becomes a point of departure for the idea that bacterial virulence and the ability to liberate proteotoxins may be correlated in some instances. In an earlier paper, it was shown that *B. pestis* became distinctly enhanced in virulence after being incubated with normal guinea-pig serum. At first it was thought that this phenomenon was limited to treatment with normal serum, but such is not the case, since heated serum had a like action. This has been demonstrated also by Rowland,<sup>3</sup> working with horse serum. The nature of this mechanism has not been defined clearly, if at all, since the function of bacteria in the production of shock has been variously explained. Recent studies by Jobling and Peterson<sup>4</sup> show that bacteria remove the serum-antiferments, thus liberating the ferments normally present in the serum and enabling them to act on the constituent proteins. This conception of the mechanism of shock does not explain entirely the ability to obtain proteotoxins by means of inactivated sera, as has been shown by Neufeld and Dold;<sup>5</sup> rather, it suggests that the thermostabile substance acts like a sensitizer and that the increased 'virulence' noted in this paper is to be explained possibly by the action of complement upon the antigen-sensitizer complex, after the bacteria have been able to multiply sufficiently by virtue of their increased resistance.

Virulence and the ability to produce proteotoxins, then, seem to be synonymous. The unusual susceptibility of the guinea-pig to plague infection may fall into line with this conception. As has been seen, *B. pestis* becomes more virulent after contact with serum from this animal. Some substance, in all likelihood normal sensitizer, is absorbed out of the serum and the combination so formed becomes amenable to the action of complement. The ease and rapidity with which proteotoxins may be produced will determine the degree of virulence by which the organism in question may be characterized.

<sup>2</sup> Jour. Exper. Med., 1914, 20, p. 582.

<sup>3</sup> Jour. Hyg., Plague Supplement, 1912.

<sup>4</sup> Jour. Exper. Med., 1914, 20, p. 480.

<sup>5</sup> Cited by Zinsser in Infection and Resistance, 1916, p. 424.



## SUMMARY AND CONCLUSIONS

Powerful shock-producing poisons can be obtained by allowing normal guinea-pig or horse serum to act upon sensitized plague bacteria.

The proteotoxins of the plague bacteria are thermostabile, resisting prolonged heating at temperatures ranging from 50-61 C.

A wide range exists within which the poisons may be obtained. The essential requirement is sufficient sensitization of the plague organisms.

Proteotoxins are obtainable even after considerable exposures. The average period used in these experiments was 14 hours, exclusive of the hour or more necessary for centrifugation and the time required during subsequent operations. In several instances a period as long as 18 hours did not preclude the formation of the toxic substances.

The mechanism of plague infection seems to depend on a primary sensitization of the organisms, the antigen-sensitizer complex being important for poison-production. Virulence, so far as *B. pestis* is concerned, is probably an altered state of the bacteria which caused poisons to be liberated when the normal serum components are allowed to act on the newly-formed complex.

# AMERICAN MORTALITY STATISTICS AND THE MILLS-REINCKE PHENOMENON \*

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In 1910, Sedgwick and MacNutt<sup>1</sup> published the results of their studies on the mortality statistics of several cities which had made a sudden change from a polluted to a pure water supply, and formulated what they termed the Mills-Reincke phenomenon and Hazen's theorem.

The city of Lawrence, Mass., installed a water filtration plant in 1893, and during the period immediately following, Hiram F. Mills, chief city engineer and a member of the Massachusetts State Board of Health, noted a marked reduction in the general death rate of the city, in addition to the decrease in the specific death rate from typhoid fever. At about the same time, the city of Hamburg, Germany, began the filtration of its water supply, and J. J. Reincke, health officer of Hamburg, emphasized in his annual reports that the reduction of the general death rate greatly exceeded the number of lives that could possibly have been saved from typhoid fever. Sedgwick and MacNutt, recognizing the sanitary significance of these observations, have applied to them the name of the 'Mills-Reincke phenomenon.'

At the end of a paper on the "Purification of Water for Domestic Use. American Practice," Hazen<sup>2</sup> discusses filtration from the standpoint of hygiene. After comparing the crude death rates of several cities which had made a radical change in their water supply with others similarly situated but which had made no such change, he expresses the opinion that, "where one death from typhoid fever has been avoided by the use of a better water, a certain number of deaths, probably two or three, from other causes have been avoided." This numerical expression, Sedgwick and MacNutt have termed "Hazen's theorem."

Both the Mills-Reincke phenomenon and Hazen's theorem have been widely quoted in public health literature, and attempts have been made to demonstrate their operation in various cities by showing a

\* Received for publication January 20, 1917.

<sup>1</sup> Jour. Infect. Dis., 1910, 7, p. 489.

<sup>2</sup> Trans. Am. Soc. Civil Eng., Internat. Eng. Cong., 1905, 54, D, p. 151.

general reduction of the death rates in the years immediately following purification of their water supplies. The paper by Sedgwick and MacNutt contains a summary of the literature up to the date of its publication. I shall confine myself to a summary of the work done since then, and to particular information bearing on my own studies.

#### HISTORICAL REVIEW

Sedgwick and MacNutt<sup>1</sup> believe that they found abundant evidence of the occurrence of the phenomena under discussion in the mortality statistics of Hamburg, Germany; Lawrence and Lowell, Mass.; and Albany and Binghamton, N. Y. Binghamton showed irregular changes in its death rates following purification of its water supply, and during the second year following filtration there was a marked rise. Watertown, N. Y., in the 3 years following improvement in its water supply, showed an actual increase in the total death rate minus the typhoid component. This is explained on the basis of a relatively imperfect purification of the water.

Lederer<sup>2</sup> has compared the decrease in the typhoid death rates and the general death rates in 8 cities which had improved their water supply during the period 1900-1908. His figures included Providence, R. I.; St. Louis, Mo.; Youngstown, O.; Ithaca, N. Y.; Paducah, Ky.; Watertown and Binghamton, N. Y.; and Paterson, N. J. He found that the average reduction in the crude death rate was 3 times as great as would be expected from the reduction in typhoid mortality alone.

In a study of the relation of polluted water supplies to infant mortality, McLaughlin<sup>3</sup> found that cities with polluted water supplies have an unduly high death rate from diarrhea and enteritis in children, during the winter and spring months. He believes that the infected water is largely responsible. A brief study of the Mills-Reincke phenomenon is also included in his publication. Cincinnati, O., showed a marked reduction in the total number of deaths during the 3 years following the operation of its filters. Columbus, O., seemed to suffer an actual increase in the number of deaths during the years immediately following filtration. The average number of deaths in Philadelphia, Pa., for a period of 3 years before and after filtration is not appreciably less than can be accounted for by the saving of lives from typhoid fever. McLaughlin suggests that this may possibly be explained by the fact that filtered water was at first supplied to only one section of the city in 1906, and the filtered water area gradually extended until 1910. In the city of Pittsburgh, Pa., for every death from typhoid fever avoided, McLaughlin found but 1.1 deaths less from all other causes. This is considerably less than Hazen's theorem suggests.

The water supply of Chicago for many years was contaminated by sewage from the Chicago River entering Lake Michigan and finding its way to the water works intakes. The result was a notoriously high typhoid fever death rate with occasional explosive epidemics. In 1900, most of this sewage was diverted into the Drainage Canal, built for the purpose and draining away from the lake. Soper, Watson and Martin<sup>4</sup> in their short discussion of the effect of opening the Drainage Canal on the mortality of Chicago, conclude

<sup>1</sup> Am. Jour. Pub. Hyg., 1910, 20, p. 295.

<sup>2</sup> Pub. Health Rep., 1912, 27, pp. 579-612.

<sup>3</sup> Report to Chicago Real Estate Board on Disposal of Sewage and Protection of Water Supply of Chicago, Ill., 1915.

that according to their figures there has been no marked fall in the general death rate following improvement in the water supply. Those specific diseases which showed a decrease seemed to be in no apparent relation to the water supply.

A comparison of the mortality statistics of Cincinnati, O., for a 3 year period before and after filtration was made by Landis.<sup>6</sup> This shows that the deaths from diarrheal diseases in children under 2 years, including inanition and convulsions, for the 3 years before filtration was 133 per 100,000 of population; for the same period following filtration, the rate was 98 per 100,000 of population. During the 3 years following efficient milk inspection, there was a further drop in the rate to 76 per 100,000. These statistics illustrate the danger of attributing a marked fall in mortality to any one factor. The death rate from acute and chronic nephritis during a 5 year period before filtration was 123 per 100,000, while for a similar period following filtration it was 136 per 100,000. The average death rates from diseases of the circulatory system during the same periods was 203 and 228 respectively, per 100,000.

Fuller<sup>7</sup> also discusses the experience of Cincinnati and gives a table showing the death rates for the same period. He finds that there are irregular but quite striking reductions in mortality from specific diseases other than typhoid fever, as well as in the total death rate.

In the report of the board of health of Columbus, O., for 1913, Louis Kahn publishes the results of his calculations which show that the average total death rate from 1901 to 1908 was 15.4, and from 1908 to 1913, 14.6 per 1000 of population, indicating that there has been a decrease in the general death rate as well as the typhoid fever rate since their filtration plant went into operation.

The report of the department of health of Pittsburgh for 1911 contains the death rates for the unfiltered area as compared with the filtered area, as indicated in Table 1.

TABLE 1  
COMPARISON OF DEATH RATES IN PITTSBURGH OF FILTERED AND UNFILTERED WATER AREAS

	Filtered Water Area (Old City and South Side)	Unfiltered Water Area (North Side)
1909	12.6	30.9
1910	13.1	47.3
1911*	11.2	45.1

\* The total number of deaths for the filtered water area during 1911 was 46; for the unfiltered area, 61.

The explanation for the reduction in mortality from the so-called 'water-borne diseases' is apparent, and this no doubt accounts for the greater part of the reduction in the general death rate following purification of a water supply. McLaughlin<sup>4</sup> thinks that the greatest measure of reduction is due to a decrease in mortality from diarrhea and enteritis in children, while Landis<sup>6</sup> is of the opinion that efficient food inspection is equally effective. No satisfactory explanation has been advanced for the mortality reduction in other diseases not thought to be to any extent water-borne, such as pneumonia, nephritis, cardiac diseases and tuberculosis. In the discussion of Hazen's paper, Jordan<sup>2</sup>

<sup>6</sup> Annual Report of Dept. of Health, Cincinnati, O., 1914.

<sup>7</sup> Sewage Disposal, 1912.

pointed out that owing to errors in diagnosis, deaths from typhoid fever are sometimes reported as death from malaria and typhomalaria,<sup>8</sup> and that at least a part of the reduction noted following the purification of a water supply is due to the disappearance of these deaths from the statistics. In a recent paper, Jordan<sup>8</sup> again draws attention to the fact that at least a part of the decrease in the non-typhoid mortality is due to the reporting of typhoid deaths under such heads as malaria or typhomalaria. He also suggests that it might be worth while to investigate more closely the relationship between deaths from the sequelae of typhoid fever and the Mills-Reincke phenomenon and Hazen's theorem. The problem is extremely complicated because the number of deaths avoided from typhoid fever, and the number of lives saved from water-borne diseases are but 2 factors in a situation involving a multiplicity of operative factors. The difficulty will be in assigning to each its proper significance, and there is danger of emphasizing some one factor to the exclusion of others equally important.

Dublin,<sup>9</sup> in an analysis of the causes of death in 54 of 1428 recovered cases of typhoid fever, found that 38.9% died of tuberculosis; 14.8% of diseases of the heart; 7.4% of pneumonia; and 7.5% of nephritis. The greatest number of deaths was found to occur within the first 2 years after recovery from typhoid fever. Comparison with tables of life expectancy showed that within the first year the actual mortality was about 3 times the expected, and in the second year more than double the expected. On the bases of this double mortality in the 3 years following recovery from typhoid fever, Dublin has estimated that 7781 deaths are annually attributable to the sequelae of this disease throughout the United States. These figures are based upon a small number of cases occurring among a selected class. Therefore, broad general conclusions based upon them are hardly justified. On the other hand, the cases were selected with great care to insure accuracy of diagnosis, and the records are complete.

A recent report<sup>10</sup> contains a chart of the death rates per 100,000 of population from certain important causes in the Registration Area during the years 1900-1914. Tuberculosis (all forms) has a curve almost identical with pneumonia (all forms) from 1900 to 1906. In the following years the curves are similar, both showing a sharp downward tendency, but pneumonia has 2 sharp peaks in 1907 and 1910,

<sup>8</sup> Jour. Am. Med. Assn., 1916, 66, p. 467.

<sup>9</sup> Am. Jour. Pub. Health, 1915, 5, p. 20.

<sup>10</sup> Bureau of the U. S. Census, Mortality Statistics, 1914, p. 23.



which do not appear in tuberculosis. The curves for organic heart diseases and nephritis have similar outlines and run almost parallel. Diarrhea and enteritis of children resembles in outline the curves for tuberculosis and pneumonia, but the sharp peaks of the latter are missing. The curve for typhoid fever slopes gradually downward, the

TABLE 2  
TOTAL DEATH RATES PER THOUSAND POPULATION DURING THE YEARS 1901-1914

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	19.9	19.1	18.3	18.3	18.5	18.6	18.6	18.3	16.8	17.2	17.1	16.4	16.4	16.1
New York.....	19.9	18.6	18.0	20.1	18.4	18.3	18.3	16.3	16.0	16.0	15.2	14.5	14.3	14.1
Cincinnati.....	19.5	17.9	18.6	20.6	18.9	20.4	18.1	18.0	16.5	17.4	16.5	16.6	16.9	16.0
Columbus.....	14.1	15.5	16.3	16.1	14.9	15.3	15.5	15.2	14.0	15.4	14.3	14.4	15.3	14.8
Philadelphia...	18.0	17.5	18.7	18.7	17.6	19.1	18.6	17.3	16.4	17.4	16.6	15.3	15.7	16.1
Pittsburgh*....	18.8	20.9	20.8	19.2	19.9	19.9	19.3	17.3	15.8	17.9	14.9	15.9	17.1	15.7

\* As now constituted.

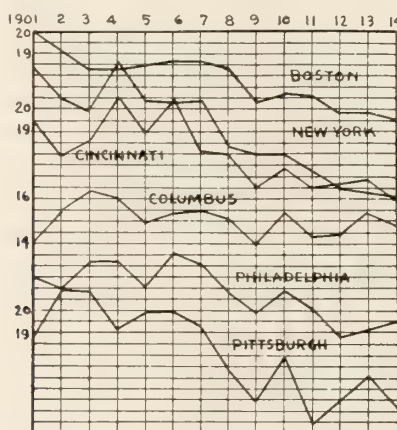


Chart 1. Total death rates per thousand of population. Plotted from Table 2.

limits being between 15 and 35 per 100,000, and shows no sharp irregularities. The year of highest mortality, 1900, is followed by a marked drop in the rates from tuberculosis, pneumonia, diarrhea and enteritis, in 1902. But, in 1904, tuberculosis and pneumonia show a sharp rise, while diarrhea and enteritis show a similar peak 2 years later, in 1906. Pneumonia, heart diseases, diarrhea and enteritis show well defined

peaks in 1907, a year following a slight rise in the typhoid fever rate. The fluctuations in the diarrhea and enteritis rate are more marked than those of typhoid, but it is noticeable that, in general outline, the 2 curves resemble each other. In typhoid fever, there is the beginning of a rise in 1901, lasting for 2 years. In the case of diarrhea and enteritis, this rise begins where the typhoid rise ceases and reaches its maximum 3 years later, in 1906.

Before proceeding with my studies, I sought to determine to what extent clinical data and experience support the facts cited regarding the sequelae of typhoid fever.

#### TUBERCULOSIS

Woodruff<sup>11</sup> studied the effect of typhoid fever on tuberculosis by making inquiries of tuberculosis workers and by comparing the curves of tuberculosis and typhoid fever death rates in different countries. The percentage of patients suffering from tuberculosis giving a history of previous typhoid fever varied from 3.6 to 17.06%. Matson in reply to Woodruff's questionnaire estimated that tuberculosis was a direct sequel to typhoid in 10% of all his cases. In the countries for which the death rates of tuberculosis and typhoid fever were compared, the curve showed that where a drop in the typhoid fever mortality occurred, this was paralleled by a drop in the tuberculosis mortality. The data for the large cities do not show such close similarity, and the explanation given is that the nonresident deaths are responsible for the discrepancy. A remarkable reduction in the incidence of tuberculosis following a drop in the typhoid fever rate was found in the experience of the British Army in India. The figures for the U. S. Army also show that with a fall in the typhoid fever rate there has been a coincident reduction in tuberculosis. The health authorities of both New York and Pennsylvania reported that typhoid and tuberculosis had markedly declined in their respective states in recent years and at about the same rate. Woodruff believed that the facts brought out by his figures serve to explain the fall in the death rate from tuberculosis following purification of public water supplies observed by Sedgwick and MacNutt,<sup>12</sup> namely, that not only are a certain number of deaths from typhoid prevented, but that the incidence of tuberculosis is reduced by removing one of the predisposing causes. Of interest in this connection may be cited the experiments of Brown, Petroff and Heise.<sup>13</sup> They studied the results of constant contamination of Saranac Lake River with sewage from the village of Saranac Lake, N. Y. Ninety % of cases of open tuberculosis of the lungs excrete viable tubercle bacilli in the feces and one would naturally expect that a stream contaminated with sewage from a tuberculous community would contain living tubercle bacilli. They found that guinea-pigs could be infected with water taken at a distance varying from the sewer's mouth to at least 3 miles down stream on cloudy days, but on sunny days the acid-fast organisms obtained 2.9 miles down stream produced no tuberculosis.

In commenting on the effect of antityphoid vaccination in the U. S. Army, Shimer<sup>14</sup> says that this practice has not increased other diseases, but that

<sup>11</sup> Am. Med., 1914, 20, p. 17; Science, N. S., 1914, 39, p. 173.

<sup>12</sup> Tuberculosis in Massachusetts, 1908, p. 181.

<sup>13</sup> Am. Jour. Pub. Health, 1916, 6, p. 1148

<sup>14</sup> Jour. Indiana Med. Assn., 1914, 7, p. 282.

tuberculosis, the disease suspected of being on the increase, has actually decreased in the army since 1908.

McCrae<sup>15</sup> thinks that it is difficult to determine by statistics whether typhoid fever predisposes to a subsequent attack of tuberculosis or whether tuberculosis confers a relative immunity against typhoid. Tuberculosis may follow typhoid fever, but in the majority of cases, it is probable that the diagnosis was wrong and the condition was tuberculous from the beginning. Acute miliary tuberculosis is not infrequently mistaken for typhoid fever.

#### THE CIRCULATORY SYSTEM

There are no changes in the heart and blood vessels peculiar to typhoid fever. The cardiovascular complications and sequelae in the cases coming to Osler's clinic for 14 years have been studied by Thayer,<sup>16</sup> who also reviewed the literature on the subject. Endocarditis is a rare complication, only 11 cases being reported among 2000 autopsies at Munich, while but 3 were found in nearly 100 autopsies at the Johns Hopkins Hospital. Thayer quotes Landouzy and Siredey as of the opinion that next to articular rheumatism, typhoid fever appears to be accompanied by more cardiovascular complications than any of the other infectious diseases.

Thayer himself was able to examine 183 of 1400 patients discharged from the typhoid fever wards of the Johns Hopkins Hospital over a period of 13 years. He found that the average systolic blood pressure was higher in these old typhoids than in healthy controls. The average size of the heart was greater than in the same cases at the time of admission to the hospital. Cardiac murmurs were heard with greater frequency than during the attack. Although the series of cases is small, there can be no doubt that it indicates that persons recovering from typhoid fever are more likely to suffer from cardiac diseases than normal persons, and, other conditions being equal, are further on the way toward arteriosclerosis.

In a study of 793 cases of typhoid fever in von Jaksch's clinic at Prague during 1889-1903, Skutezky<sup>17</sup> reports myodegeneration of the heart in 6 cases, of which 5 died; pericarditis, 1 case, with recovery; endocarditis, 15 cases, of whom 6 died; embolism, 3 cases, all ending fatally; thrombosis, 19 cases and 2 deaths.

#### PNEUMONIA

Pneumonia may not infrequently be a complication of typhoid fever and may be the cause of death during the acute stage, but pneumonia developing some time after recovery can very rarely be attributed to typhoid fever. Bronchitis often associated with typhoid fever may predispose to pneumonia, and if it becomes chronic may end in tuberculosis. Of the cases in Skutezky's series which showed respiratory involvement, bronchitis was the most frequent, and occurred in 38.0% of all cases. Next in order of frequency came laryngitis in 1.5%, and lobar pneumonia in 5.5%, half of which ended fatally. Pulmonary tuberculosis followed in 1.5%, and of these 41.6% died.

True nephritis is considered a rare complication of typhoid fever by Herrick,<sup>18</sup> who quotes Bartels as having seen but 2 instances in 1000 cases. The involvement of the kidneys is usually transient, but during convalescence bacilli are excreted through the kidneys and a nephritis may develop just as

<sup>15</sup> Osler's Modern Medicine, 1907, 2, p. 179.

<sup>16</sup> Am. Jour. Med. Sc., 1904, 127, p. 391.

<sup>17</sup> Ztschr. f. Heilk., 1906, 27, p. 14.

<sup>18</sup> Osler's Modern Medicine, 1909, 6, p. 109.

in any other acute infectious disease. Of Skutezky's cases, 3.2% were complicated by acute nephritis and 38.4% died. Chronic nephritis followed in only 1.01%, with 37.5% mortality. Selby<sup>19</sup> states that albuminuria is not an infrequent accompaniment of typhoid fever, and quotes Stolte as having found this condition in 60% of 371 cases. According to Selby, also, acute parenchymatous nephritis occurred in 3% of Talley's series of 18,000 cases.

From this brief review of the literature it appears that typhoid fever may definitely predispose to tuberculosis. During the acute stages and during the long convalescent period which often follows, the resistance to tuberculosis is lowered. Then, too, under such conditions, a latent tuberculous focus is likely to become active. Just how large a percentage of the tuberculous patients who give a history of typhoid fever actually suffered from typhoid, and the exact relation between the typhoidal attack and the subsequent tuberculosis is difficult to determine. Errors in the diagnosis of typhoid fever are frequently made, and there is still a tendency among physicians to call any continuous indefinite fever, typhoid fever. Such errors of diagnosis are less likely to occur in large cities where laboratory facilities are available.

Respiratory infections are not uncommon complications of typhoid fever and a bronchitis may lead to tuberculosis. That a reduction in the typhoid fever death rate is accompanied by a coincident reduction in the death rate from tuberculosis is indicated by the studies of Woodruff, and the experiences of the U. S. Army and the British Army following antityphoid vaccination. The relation of the 2 diseases is worthy of further clinical investigation.

That the heart and blood vessels are affected in typhoid fever is shown by clinical experience, but the frequency with which permanent damage results is not yet clearly established.

The kidneys may suffer during an attack of typhoid fever as in any of the acute infections; the statistics available are not large enough to determine approximately how often nephritis follows typhoid fever. There is a feeling among medical authorities that true nephritis following typhoid fever is rare.

#### STATISTICAL STUDIES OF SEVERAL CITIES

A review of the literature indicates an agreement among those discussing the question, that further investigation of the effect of water supply upon diseases other than typhoid fever is necessary. As far as

<sup>19</sup> Am. Jour. Med. Sc., 1908, 135, p. 224.



I am aware, no detailed studies have been made of the mortality statistics of the larger cities extending over a period of years. It therefore seemed desirable to make such a study of several cities where a sudden change had been made from a polluted to a pure water supply to determine whether the Mills-Reinke phenomenon and Hazen's theorem are applicable here as well as in those cities studied by Sedgwick and MacNutt. If the former is based on sound scientific principles, as the authors claim, it should be operative wherever there has been a marked drop in the typhoid fever death rate owing to the substitution of a safe for a polluted water supply. If this phenomenon should be found not to occur in every situation where it might be expected, such a study ought to reveal the reasons.

As examples of cities where a sudden change from a polluted to a pure water supply had been made, Chicago, Cincinnati and Columbus, O., Philadelphia and Pittsburgh have been selected. The detailed mortality statistics for a definite period before and after the change in water supply have been tabulated. Similar figures for New York City and Boston, Mass., covering the same years have served as controls. In addition to a comparison of the crude death rates with the death rates from typhoid fever, malarial fever and diarrhea and enteritis, the death rates of those diseases known to be sequelae of typhoid fever have been especially studied. Curves of the death rates from pulmonary tuberculosis, pneumonia, nephritis and heart disease have also been plotted to see whether during the 3 years immediately following years of high typhoid mortality, there could be demonstrated a sudden rise in mortality from these diseases, a possibility which the data published by Dublin would seem to suggest.

Chicago\* made a sudden and radical improvement in its water supply by building the Drainage Canal which reversed the flow of the Chicago River and prevented, to a great extent, the contamination of its water works intakes in Lake Michigan. The canal was opened in 1900. A rapid sand filtration plant was placed into service in Cincinnati in 1908, and in Columbus, during the latter part of the same year. Both Philadelphia and Pittsburgh employ the slow sand filtration process. At Philadelphia, there are 5 separate plants for filtering

\* A detailed description of the Chicago water works system and a résumé of its history is given by John Ericson, in the Jour. of the Western Soc. of Engineers, 1913. Similar descriptions of the water filtration plants in the other cities studied are contained in the publications of the respective water works departments, also in a paper by Geo. A. Johnson, "The Purification of Public Water Supplies," Water-Supply Paper No. 315, U. S. Geological Survey, 1913, and Jour. Am. Water Works Assn., 1914, 1, p. 31.

We are concerned, here, only with the method of water purification and the year when it was begun.



Delaware and Schuylkill River water. The first of these to be placed in operation was the Lower Roxborough plant, in 1902. Approximately one-half the population was receiving filtered water in 1908. The Queen Lane filters, the last to be constructed, went into service November 29, 1911. The entire city, however, had begun to receive filtered water on February 28, 1909. Pittsburgh began the filtration of its public water supply in November, 1907, and by October of the next year the entire old city, representing about three-fifths of the total population, was using filtered water. The supply was extended to the south side early in 1909, adding less than one-fifth of the population to those already using filtered water. The north side, representing what was formerly the city of Allegheny, and including about one-fourth the entire city's population began to receive filtered water during the latter part of March, 1914.

New York City<sup>20</sup> began the use of chlorination in 1911, but this was only to guard against possible pollution of the Croton supply. During the period 1901 to 1914, inclusive, which has been studied, there have been no striking changes in the typhoid fever death rates. Radical changes were made in the water supply of Boston<sup>21</sup> in 1898, but these were for the purpose of increasing its capacity rather than because of gross pollution. Whatever effect this may have had on the mortality statistics of Boston probably disappeared by 1901, the earliest year employed in the calculations. No marked fluctuations in typhoid fever death rates appear during 1901 to 1914. It may, therefore, safely be assumed that New York and Boston represent suitable controls of the cities which have been studied. The mortality statistics for the various cities studied are contained in Tables 2-13. The statistics, which do not include stillbirths, are taken from corrected mortality statistics, U. S. Bureau of the Census, 1909-1914.

#### HAZEN'S THEOREM

Table 12 has been compiled from the figures published by the U. S. Census Bureau and the reports of the Chicago health department, and shows the average total number of deaths before and after filtration, and during the first and second periods in the control cities. This was the method employed by McLaughlin, but is only of value when using 1 or 2 years before and after filtration, as a comparison. For example,

<sup>20</sup> Eng. News, 1916, 76, p. 438.

<sup>21</sup> Water Supply and Work of the Metropolitan Water District in the Commonwealth of Massachusetts, 1900.

TABLE 3  
DEATH RATES FROM TYPHOID FEVER PER HUNDRED THOUSAND OF POPULATION DURING  
THE YEARS 1901-1914

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	23.8	22.1	20.5	23.6	20.8	21.2	10.2	24.7	13.8	11.3	8.7	8.0	8.2	9.0
New York.....	20.6	20.3	17.1	16.8	16.0	15.2	17.1	11.9	12.1	11.6	10.9	9.6	7.0	6.3
Cincinnati.....	54.7	61.5	42.2	79.2	40.4	70.2	45.4	18.2	13.3	8.8	11.4	7.7	6.8	6.2
Columbus.....	47.4	36.2	36.4	141.4	80.8	35.0	35.8	102.4	19.6	18.1	13.9	19.6	19.1	13.2
Philadelphia...	34.5	47.1	72.3	54.7	50.8	74.3	60.3	35.2	22.3	17.5	14.6	12.8	15.7	7.6
Pittsburgh.....	119.5	136.1	132.7	135.9	106.6	141.1	131.2	48.9	24.6	27.8	25.6	13.1	19.5	15.0

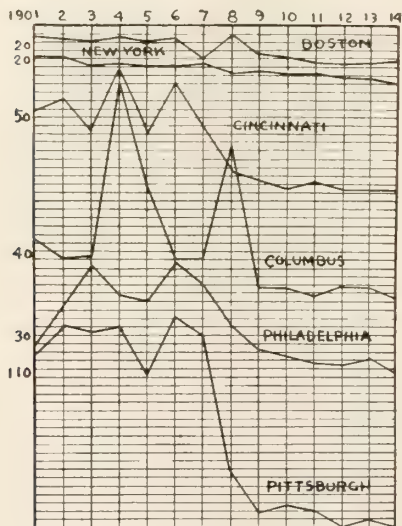


Chart 2. Typhoid fever death rates, per hundred thousand of population. Plotted from Table 3.

TABLE 4

TOTAL DEATH RATES MINUS TYPHOID COMPONENT PER THOUSAND OF POPULATION, DURING THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO, CALCULATED FROM TABLES 2 AND 3

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	19.6	18.9	18.1	18.0	18.2	18.3	18.5	18.0	16.6	17.0	16.9	16.2	16.3	16.0
New York.....	19.6	18.3	17.7	19.9	18.2	18.1	18.0	16.1	15.8	15.8	15.0	14.3	14.2	14.0
Cincinnati.....	18.9	17.3	18.1	19.7	18.5	19.7	17.6	17.8	16.3	17.2	16.3	16.5	16.8	15.9
Columbus.....	13.6	15.1	15.9	14.7	14.0	14.9	15.1	14.1	13.8	15.2	14.1	14.1	15.0	14.6
Philadelphia...	17.6	17.0	18.0	18.1	17.0	18.3	18.0	16.9	16.1	17.2	16.4	15.1	15.5	16.0
Pittsburgh.....	18.0	19.9	19.7	17.9	18.6	18.4	17.9	16.8	15.5	17.6	14.6	15.7	16.9	15.5

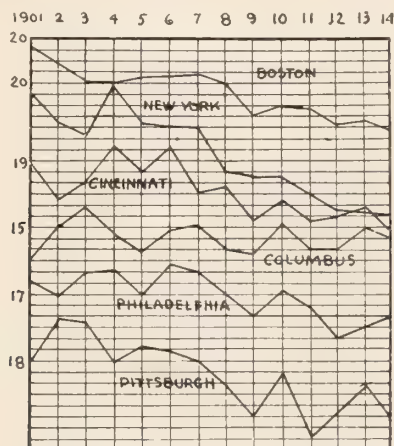


Chart 3. Total death rates per thousand of population, minus typhoid component. Plotted from Table 4.

TABLE 5

DEATH RATES FROM MALARIAL FEVER PER HUNDRED THOUSAND OF POPULATION DURING THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	1.6	1.4		0.5	0.5	0.3	1.3	0.5		0.1	0.4	0.1		
New York.....	5.0	3.5	2.0	2.7	1.4	1.8	1.7	0.7	0.9	0.7	0.7	0.5	0.3	0.3
Cincinnati.....	4.6	4.2	1.2	0.9	0.6	1.7	0.6	0.8	1.4	0.3	0.5	0.3	0.8	0.7
Columbus.....	5.4		1.4	2.8				0.6	0.6		1.1	0.5		
Philadelphia...	1.2	1.5	0.9	1.2	1.3	0.7	0.8	0.7	0.5	0.5	0.7	0.4	0.4	0.5
Pittsburgh.....	2.1	1.7	1.1		1.1		0.8	0.2		0.4		0.4	0.4	

TABLE 6

DEATH RATES FROM DIARRHEA AND ENTERITIS (UNDER TWO) PER HUNDRED THOUSAND OF POPULATION DURING THE YEARS 1901-1914, FOR THE CITIES STUDIED EXCEPT CHICAGO \*

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	116.7	106.1	106.4	98.9	108.3	91.1	86.9	112.5	103.9	100.6	106.4	84.8	72.9	64.6
New York.....	164.5	134.5	119.8	154.8	151.3	145.8	150.9	137.2	114.8	123.1	92.6	82.4	73.5	67.1
Cincinnati.....	75.3	72.9	74.6	94.8	70.2	104.1	74.7	84.1	78.2	90.1	64.3	67.9	64.5	60.2
Columbus.....	35.2	49.5	37.8	55.9	44.7	46.0	49.0	31.1	49.2	63.0	37.4	30.4	41.1	45.5
Philadelphia...	83.3	74.6	90.5	111.3	122.0	150.6	127.7	122.1	117.2	150.3	118.0	93.4	100.6	107.4
Pittsburgh.....	150.6	170.1	195.5	175.1	166.2	191.9	178.0	157.7	139.1	177.3	130.3	113.5	134.8	103.7

\* After the second revision of the international classification of causes of death in 1910, the reports from the U. S. Bureau of the Census gave the rates for diarrhea and enteritis under 2, instead of for all ages, as had been done previously. To make the figures comparable, the rates for diarrhea and enteritis under 2, for the years 1901-1909, inclusive, have been calculated from the number of deaths and population statistics contained in these reports.

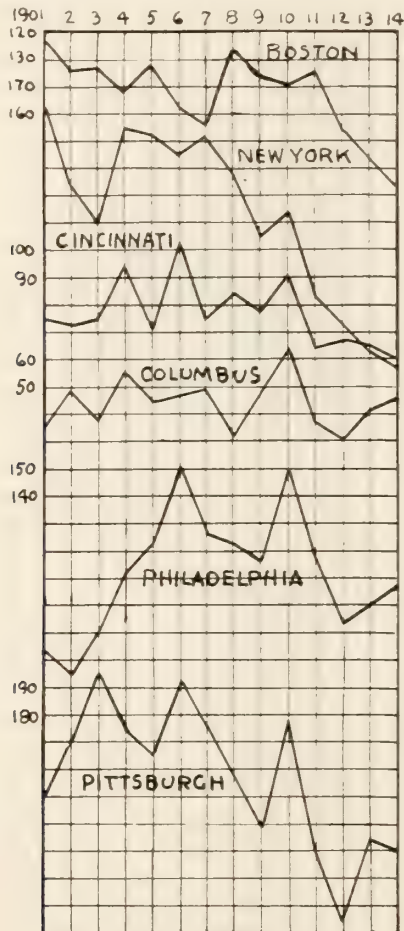


Chart 4. Death rates from diarrhea and enteritis (under two). Plotted from Table 6.

TABLE 7

DEATH RATES FROM PNEUMONIA (ALL FORMS) PER HUNDRED THOUSAND OF POPULATION FOR THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO \*

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	187.1	194.9	201.7	201.0	194.2	189.4	197.2	180.0	182.0	213.0	207.1	203.1	196.1	190.7
New York.....	264.1	264.2	251.2	310.2	232.6	249.1	263.4	204.8	230.7	222.9	209.6	194.7	194.3	180.1
Cincinnati.....	176.3	149.1	160.3	207.9	166.6	165.8	152.9	158.1	143.6	159.5	125.9	153.5	151.3	133.5
Columbus.....	105.6	126.3	150.5	141.5	143.6	138.6	133.1	106.1	119.1	135.9	121.2	119.2	113.8	114.4
Philadelphia...	192.3	196.8	182.0	183.7	134.2	161.7	161.0	148.9	138.4	167.1	159.0	133.3	145.4	167.7
Pittsburgh.....	245.5	304.1	267.2	234.8	262.7	244.5	260.3	240.3	252.4	323.7	207.2	261.1	283.3	240.4

\* The figures for the years 1901-1909, inclusive, have been calculated from the number of deaths and population statistics as published by the Bureau of the Census.

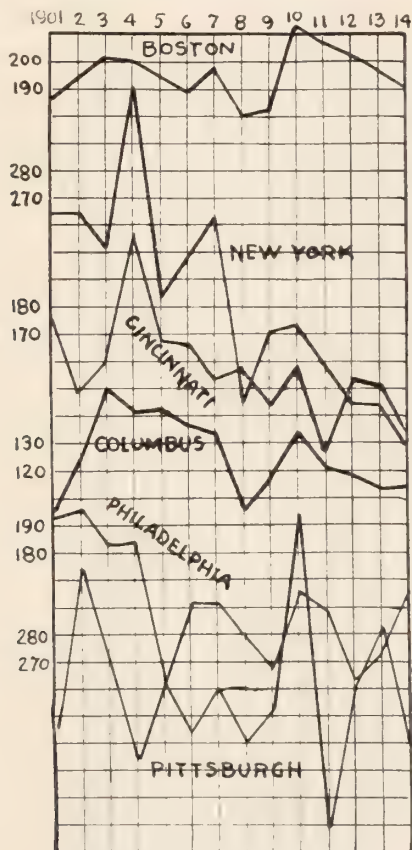


Chart 5. Death rates from pneumonia (all forms). Plotted from Table 7.



TABLE 8

DEATH RATES FROM PULMONARY TUBERCULOSIS PER HUNDRED THOUSAND OF POPULATION FOR THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	234.4	212.3	205.1	215.8	201.6	195.6	182.9	169.6	158.5	171.7	152.6	152.4	145.5	140.2
New York.....	228.8	207.8	211.6	220.4	211.1	214.0	205.8	196.6	185.6	185.0	180.9	173.4	170.1	172.6
Cincinnati.....	235.5	205.5	235.5	265.4	248.0	266.1	231.8	247.1	234.0	261.7	237.0	224.2	229.4	219.8
Columbus.....	203.5	191.2	209.7	204.9	189.0	198.7	181.5	172.0	155.3	177.5	159.1	145.5	137.4	137.4
Philadelphia...	210.9	198.4	216.2	230.4	203.4	225.1	220.8	201.7	189.0	193.5	193.5	170.2	165.0	167.7
Pittsburgh...	129.2	131.0	136.9	149.5	149.1	126.2	112.4	114.2	109.0	104.2	106.0	100.4	106.5	109.2

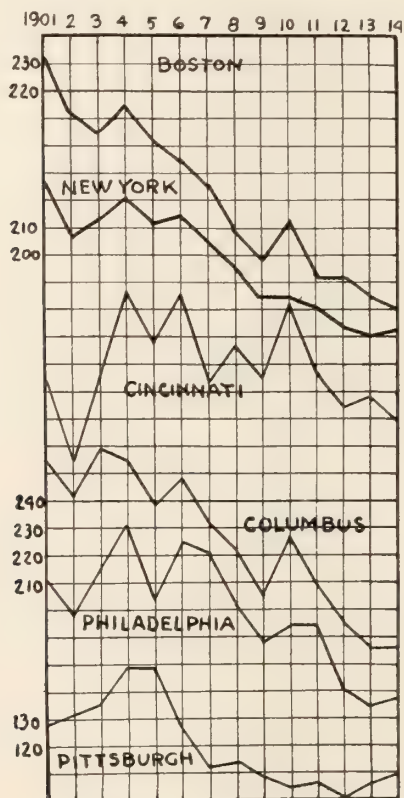


Chart 6. Death rates from pulmonary tuberculosis. Plotted from Table 8.

TABLE 9

DEATH RATES FROM ORGANIC DISEASES OF THE HEART PER HUNDRED THOUSAND OF POPULATION  
FOR THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO \*

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	179.4	199.0	193.0	191.3	198.5	196.0	210.5	181.2	196.7	201.1	189.9	205.0	211.4	218.7
New York.....	130.5	133.9	135.2	155.6	147.9	153.9	158.2	145.7	149.2	137.4	140.1	143.2	144.5	145.8
Cincinnati.....	134.0	145.2	131.4	154.7	151.7	160.7	176.6	170.8	166.1	182.6	185.7	210.6	207.8	191.7
Columbus.....	94.1	115.2	107.0	137.3	146.9	127.6	126.2	145.2	165.3	165.5	146.3	159.4	150.9	137.4
Philadelphia...	129.5	142.9	161.5	171.4	165.7	171.2	183.1	166.0	170.8	173.1	181.1	182.1	170.4	196.6
Pittsburgh.....	94.5	91.1	96.6	102.9	110.8	111.8	119.4	114.1	114.5	125.9	118.2	126.8	126.2	118.8

\* "Organic Diseases of the Heart" of the reports of the U. S. Bureau of the Census after 1910 corresponds to "Heart Disease," the title in the first revision of the International List of Causes of Death. The former, however, includes International Title 78, "Acute Endocarditis." The figures in the table for 1901-1909, inclusive, represent the rates for the 2 combined as calculated from the number of deaths and population statistics of the reports of the Bureau of the Census.

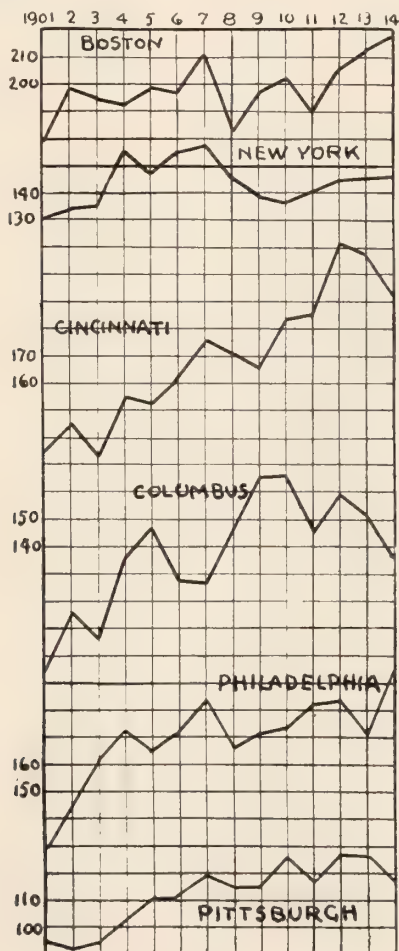


Chart 7. Death rates from organic diseases of the heart. Plotted from Table 9.

TABLE 10

DEATH RATES FROM BRIGHT'S DISEASE AND NEPHRITIS PER HUNDRED THOUSAND OF POPULATION  
FOR THE YEARS 1901-1914, FOR THE CITIES STUDIED, EXCEPT CHICAGO

City	1901	1902	1903	1904	1905	1906	1907	1908	1909	1910	1911	1912	1913	1914
Boston.....	82.6	79.0	85.5	81.9	82.6	90.0	86.7	85.6	86.2	89.5	98.9	93.0	91.9	90.7
New York.....	162.1	155.4	159.4	171.0	160.5	163.6	164.7	140.9	145.5	142.6	142.8	140.3	138.1	139.9
Cincinnati.....	121.6	116.6	122.8	130.1	121.0	143.0	126.6	125.8	142.2	137.3	144.9	164.1	160.1	145.9
Columbus.....	60.4	62.7	74.2	51.7	60.8	75.1	95.5	80.5	81.2	79.5	78.0	79.5	94.3	83.6
Philadelphia...	137.5	135.8	149.3	159.2	164.8	167.6	176.3	159.4	158.9	171.9	164.5	159.5	177.5	174.4
Pittsburgh.....	54.5	63.8	57.2	54.2	59.7	62.3	72.4	70.0	75.1	74.7	73.7	79.7	82.3	88.7

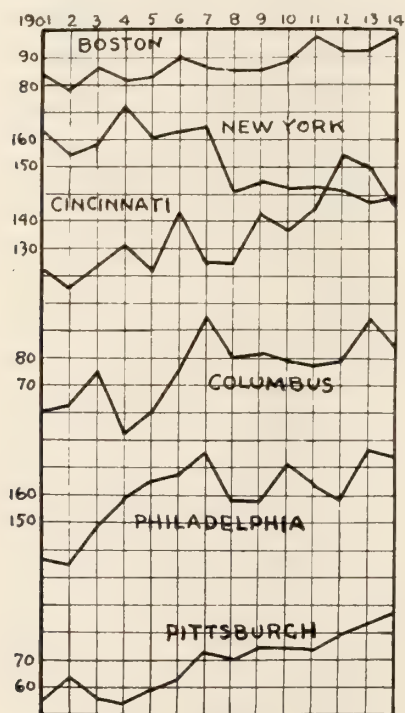


Chart 8. Death rates from Bright's disease and nephritis. Plotted from Table 10.

TABLE 11

TOTAL DEATH RATES PER THOUSAND AND DEATH RATES PER HUNDRED THOUSAND FROM CERTAIN SPECIFIC CAUSES, CHICAGO, FOR THE YEARS 1884-1915 \*

Year. Before Opening Canal	Total Death Rates	Typhoid Fever	Total Minus Typhoid	Malarial Fever	Diar- rheal Diseases (all ages)	Pneu- monia (all forms)	Tuber- culosis (all forms)	Heart Disease	Bright's Disease and Nephritis
1884	19.80	56.2	19.24	15.5	307.0	113.2	188.1	61.6	37.0
1885	18.76	74.6	18.02	16.7	254.6	110.7	193.8	69.2	35.3
1886	19.47	68.6	18.79	16.8	240.3	125.1	195.7	64.4	42.6
1887	20.27	50.3	19.77	11.4	268.6	132.6	202.6	72.2	42.1
1888	19.65	46.7	19.17	12.0	253.3	137.7	201.3	77.1	41.1
1889	18.12	48.4	17.64	11.2	251.9	125.0	181.0	71.0	39.9
1890	19.87	91.6	18.96	11.0	242.6	188.5	201.9	74.2	46.3
1891	24.16	173.8	22.43	12.4	273.8	251.8	208.9	80.9	51.7
1892	21.85	124.1	20.61	11.6	233.5	199.8	198.6	88.2	50.8
1893	21.61	53.5	21.08	6.6	260.6	196.1	211.2	88.3	60.1
1894	18.26	37.5	17.89	2.6	270.7	116.1	191.0	79.3	52.0
1895	17.72	37.9	17.35	4.1	217.0	172.6	180.2	99.8	51.3
1896	16.29	52.6	15.77	2.2	206.9	150.0	186.8	90.3	57.3
1897	14.63	29.3	14.34	1.6	165.3	144.3	172.6	91.6	62.8
1898	14.64	40.8	14.24	1.7	142.8	159.1	181.7	89.8	67.5
1899	15.68	27.2	15.41	1.5	154.8	211.4	178.9	91.7	72.6
* Canal opened 1900.									
1900	14.68	19.8	14.49	1.7	130.6	199.5	173.9	111.4	65.7
1901	13.93	29.1	13.44	1.8	126.3	178.5	164.0	111.1	57.9
1902	14.69	44.5	14.25	1.2	123.6	190.6	165.4	116.5	69.8
1903	15.62	31.8	15.31	1.1	118.4	250.1	182.5	113.0	81.9
1904	13.85	19.6	13.66	0.3	110.8	217.8	186.5	107.5	87.3
1905	13.96	16.9	13.80	0.8	129.1	184.1	188.8	108.3	103.5
1906	14.54	18.5	14.36	0.4	113.1	202.5	192.0	109.8	106.5
1907	15.72	18.2	15.54	0.3	119.6	232.1	197.2	113.2	121.9
1908	14.49	15.8	14.34	0.5	146.6	167.4	186.7	128.1	102.0
1909	14.58	12.6	14.46	0.4	145.1	219.6	181.0	147.4	105.9
1910	15.14	13.7	15.01	0.5	159.8	240.8	178.0	151.4	113.9
1911	14.49	10.7	14.39	0.4	132.8	219.5	165.9	152.9	109.5
1912	14.85	7.6	14.76	0.2	133.9	213.1	163.9	152.0	103.5
1913	15.06	10.6	14.96	0.3	137.1	207.6	164.9	147.8	99.1
1914	14.19	6.9	14.13	0.4	126.5	170.3	168.2	162.9	83.8
1915	14.18	5.4	14.13		106.5	155.8	170.3	158.6	88.0

\* Figures copied from chronological summaries of mortality statistics in the annual reports of the Department of Health of the City of Chicago for the years 1900, 1907-1910 and Bulletin Chicago School of Sanitary Instruction, February 5, 1916. Malaria figures after 1906 are from reports of the Bureau of the Census. Total minus Typhoid Rates are calculated from Columns 1 and 2.

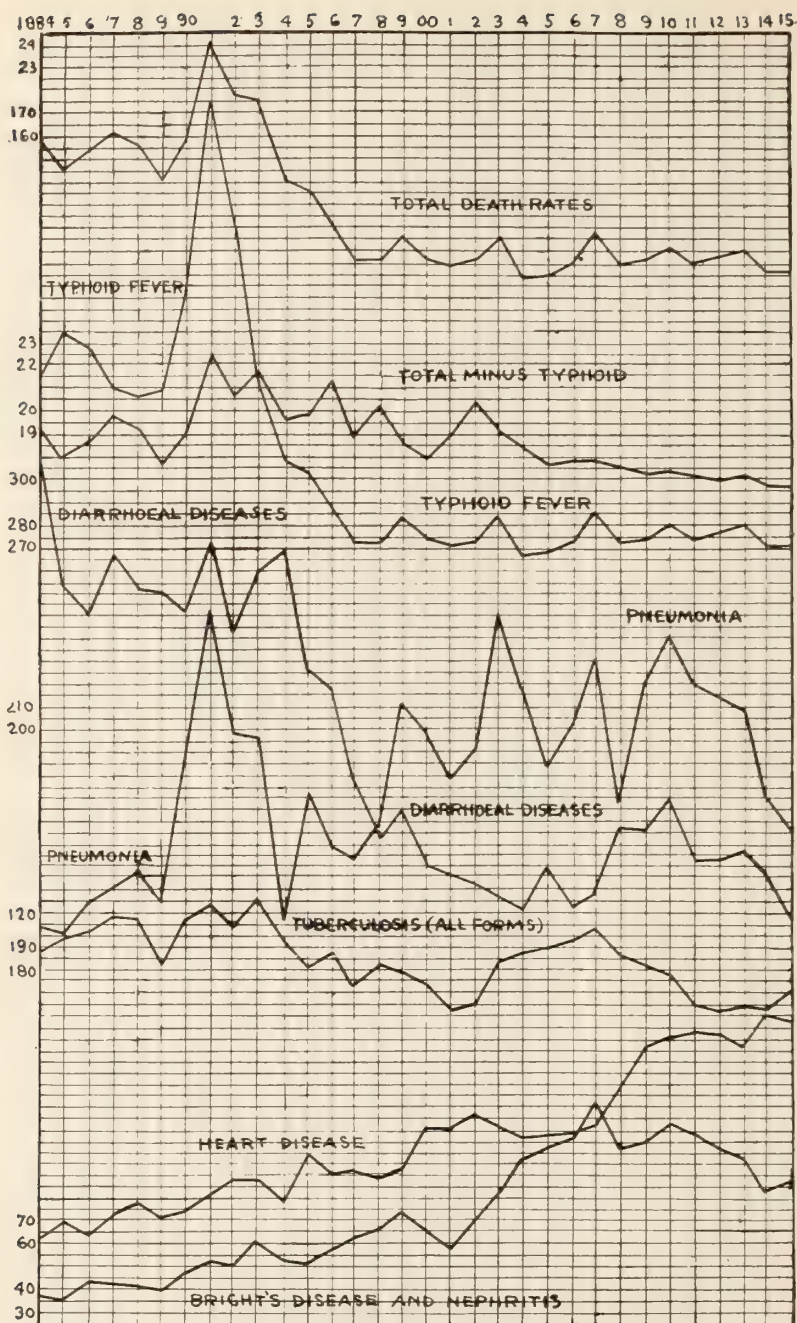


Chart 9. Total death rates per thousand and death rates per hundred thousand, from certain specific causes, Chicago, during the years 1884-1915. Plotted from Table 11.



TABLE 12

AVERAGE TOTAL NUMBER OF DEATHS IN THE CITIES STUDIED, AND AVERAGE NUMBER OF DEATHS FROM TYPHOID FEVER AT PERIODS INDICATED

City		Total Deaths	Typhoid Fever
Boston.....	First period.....	11,114	119
	Second period.....	11,635	81
	Increase.....	521	Decrease 38
New York.....	First period.....	73,379	684
	Second period.....	74,563	482
	Increase.....	1,184	Decrease 202
Chicago.....	Before canal opened.....	20,697	499
	After canal opened.....	28,189	347
	Increase.....	7,492	Decrease 152
Cincinnati.....	Before filtration.....	6,562	192
	After filtration.....	6,364	38
	Decrease.....	198	Decrease 154
Columbus.....	Before filtration.....	2,233	85
	After filtration.....	2,735	52
	Increase.....	502	Decrease 33
Philadelphia.....	Before filtration.....	25,661	791
	After filtration.....	25,877	280
	Increase.....	216	Decrease 516
Pittsburgh.....	Before filtration.....	9,444	610
	After filtration.....	8,892	131
	Decrease.....	552	Decrease 476

Calculated from mortality statistics published by U. S. Bureau of the Census, except in the case of Chicago which were obtained from the publications of the Chicago health department.

there were 7,492 more deaths after the opening of the Drainage Canal in Chicago than there were during a corresponding period before. The reasons for this large increase are that the periods under consideration are long, and include years of rapid increase in population, no account of which is taken in calculating the total number of deaths.

The figures, bearing in mind the objections mentioned, show that there has been an increase in the average total number of deaths during the second period in all the cities except Cincinnati and Pittsburgh. This is to be expected because of a steady increase in population in such large cities. The increase has been greatest in New York, the city with the largest population. The data for Chicago are not comparable with the others, because they cover a much longer period. The

smallest increase occurred in Philadelphia. Pittsburgh shows a remarkable decrease, amounting to more than the increase in Boston. No explanation can be attempted without a study of the specific death rates. The decrease in Cincinnati is only slightly greater than the number of deaths less from typhoid fever.

The decrease in the number of deaths from typhoid fever is of interest. It is greatest in Philadelphia, and yet Philadelphia did not have nearly as high a typhoid fever death rate before filtration as Pittsburgh, and a lower average rate than Cincinnati and Columbus. New York, without a sudden or radical change in its water supply, avoided more deaths from typhoid fever than any of the other cities, except Philadelphia and Pittsburgh. Among all the cities, there is considerable difference in population, and the only fair comparison would be one of rates per 100,000. This method of calculation is open to grave sources of error even in comparing the year before filtration with the year immediately following, in that either may easily happen to be a year containing an unusually large number of deaths from accidental causes, or from an epidemic of one of the infectious diseases such as influenza or pneumonia. In either case, the results would be misleading.

Table 13 shows the average total death rates and the death rates from certain specific diseases during the period before improvement of the water supply, in those cities where such an improvement was made, and for a period covering the same years in the control cities, compared with the corresponding average for a similar period following the change in water supply, and the same period in the control cities. This table was calculated from the data contained in Tables 2-11.

For Boston, the reduction in the total death rate during the second period under consideration has been 1.8, while the reduction in the typhoid fever mortality has been 0.08 per 1000, showing that for every death less from typhoid fever in Boston there have been approximately 22 deaths less from other causes. In New York, the reduction in total mortality compared with the reduction in typhoid mortality is about 51:1. During the period following the improved water supply in Chicago, the reduction in the general death rate was 3.6, while the reduction from typhoid fever was 0.07 per 1000, giving a saving of about 13 lives from other causes to every death less from typhoid fever. The data for Chicago cover a much longer period than those of the other cities, and it is probable that the figures for a shorter period would have shown a smaller reduction in the general death rate and a

greater reduction in the typhoid fever rate, making the relative number of deaths avoided from other causes lower. Cincinnati shows a reduction in total mortality of 2.3 per 1000, and a reduction in typhoid mortality of 0.45, indicating that for every death from typhoid fever

TABLE 13  
AVERAGE TOTAL DEATH RATES PER THOUSAND, AND AVERAGE DEATH RATES PER HUNDRED THOUSAND OF POPULATION FROM CERTAIN SPECIFIC DISEASES FOR THE CITIES STUDIED \*

City	Total Death Rates	Typhoid Fever	Malarial Fever	Diarrhea and Enteritis	Pneumonia	Tuberculosis	Heart Disease	Nephritis
Boston	18.7	20.3	0.8	102.0	180.8	206.8	195.4	84.0
	16.9	11.9	0.1	92.2	196.0	155.8	200.5	92.1
	1.8	8.4	0.7	9.8	15.2†	51.0	5.1†	8.1†
New York	18.8	17.6	2.6	145.9	262.1	214.2	145.0	162.4
	15.2	9.9	0.6	98.7	205.3	180.6	143.7	141.1
	3.6	7.7	2.0	47.2	56.8	33.6	1.3	21.3
Chicago	18.8	50.8	8.7	221.5	158.4	192.1	80.6	50.6
	14.6	17.5	0.6	120.6	203.1	114.0	139.7	94.4
	4.2	33.3	8.1	100.9	44.7†	78.1	50.1†	43.8†
Cincinnati	19.1	56.2	1.9	80.9	168.4	241.1	150.6	125.9
	16.8	10.3	0.8	72.7	146.5	236.1	187.9	145.6
	2.3	45.9	1.1	8.2	21.9	5.0	37.3†	19.7†
Columbus	15.3	64.4	1.3	43.6	130.6	193.8	124.9	70.1
	14.7	17.2	0.4	44.4	120.6	152.0	154.1	82.7
	0.6	47.2	0.9	0.8†	10.0	41.8	29.2†	12.6†
Philadelphia	18.3	54.8	1.1	108.5	173.1	215.0	160.7	155.8
	16.4	17.9	0.5	114.1	151.4	182.9	177.1	166.6
	1.9	36.8	0.6	5.6†	21.7	32.1	16.4†	19.8†
Pittsburgh	19.8	129.0	0.9	175.3	259.9	133.4	103.8	60.6
	16.3	24.9	0.2	136.6	258.3	107.0	120.6	77.7
	3.5	104.1	0.7	38.7	1.6	26.4	16.8†	17.1†

\* The first row of figures represents the period before filtration or change in water supply in those cities where such a change was made, and for the first half of the period under consideration in the control cities. In the second row are the figures for the second period, while the third row represents the difference. The classification of diseases corresponds with that in Tables 2-11.

† Represents an increase. All other third row figures indicate a decrease. Calculated from Tables 2-11.

avoided there have been approximately 5 lives saved from other causes. During the period following filtration in Columbus, there has been a reduction in the general mortality amounting to 0.6 per 1000, while the

reduction in typhoid mortality has been 0.36, or a saving of less than 2 lives from other causes for every 1 from typhoid fever. Columbus has had a low general death rate throughout the entire period before and after filtration. In Philadelphia, the reduction in the total death rate after filtration was 1.9 per 1000, while the reduction in the typhoid fever rate was 0.36, indicating that for every death avoided from typhoid fever, about 5 deaths from other causes have been avoided. The reduction in total death rate in Pittsburgh following filtration was 3.5 per 1000, and the reduction in typhoid fever rate was 1.04, so that for every death avoided from typhoid fever about 3 deaths from other causes have been avoided.

A comparison of the figures shows that in those cities where a sudden change has been made from a polluted to a pure water supply, the reduction in the general mortality compared with the reduction in typhoid mortality varies from 2:1 to 13:1. In Boston, the ratio was 22:1, and in New York, 51:1. The comparative reduction was 4 times as great in New York, one of the control cities, as in Chicago, where the greatest reduction occurred among the cities with an improved water supply. The comparison, however, is neither fair to New York or the other cities. The figures for Chicago cover a period of 32 years, during which there has been a gradual reduction in the general death rate, while the figures for New York and the other cities cover only a period of 14 years.

In both control cities there has been a gradual reduction in the general death rate, which compares favorably with the reductions observed in those cities which have made a sudden improvement in their water supplies. The average reduction in New York was greater than in any of the other cities except Chicago, and the figures of the latter include a much longer period. The average reduction in Boston was greater than in Columbus. Yet neither New York nor Boston made radical changes in its water supply during the periods under consideration. The average typhoid fever death rates in the control cities during the second period are less than the average rates in any of the other cities during the period following improvement in water supply, with the exception of Cincinnati which is lower than Boston. It is therefore evident that in the control cities without a marked reduction in typhoid mortality there was, however, a reduction in the general mortality exceeding, in some cases, the reduction shown by cities which had made a sudden change from a polluted to a pure water supply.



## THE MILLS-REINCKE PHENOMENON

In considering the reduction from specific diseases, the reduction in the death rates from typhoid fever is the most marked and most constant. Detailed studies are already on record for the various cities under consideration and have demonstrated the relation between high typhoid incidence and polluted water supply. The greatest average reduction occurred in Pittsburgh; the smallest, in Chicago. It is of interest to note that of the 5 cities studied, Cincinnati has the more closely approached the 'residual' rate as shown by Boston and New York during the period following filtration.

During the second period in Chicago, the death rate from 'reported' malaria, which was high for a nonmalarial region, was reduced to a rate equal to that in New York. In the other cities, the reductions are no more striking than in the controls.

Diarrhea and enteritis under 2, show a fall in every case except Columbus and Philadelphia. In Boston, the reduction has been slight. New York, without a radical change in its water supply, shows a greater reduction in its rate from diarrhea and enteritis than any of the other cities with which it is comparable.

It is difficult to obtain a comparable set of statistics for pneumonia, since the question of diagnosis as well as the age distribution of the population are important factors in determining the death rate. Changes in classification of the causes of death also affect the figures for pneumonia. The statistics, which include those of all forms of pneumonia, show that there has been an appreciable reduction in Columbus, Philadelphia, and New York; a decrease not nearly so large in Cincinnati, practically no reduction in Pittsburgh, and a definite increase in Boston. In this case, one of the control cities shows the most marked decrease, while the other shows the greatest increase. Such differences are to be explained by the differences in age and sex distribution of the population.

The greatest average reduction in pulmonary tuberculosis occurred in Boston. New York shows a greater reduction than Philadelphia and Pittsburgh. Cincinnati showed very little reduction during the period following filtration, and a much higher average rate during the second period than any of the other cities studied. In Columbus and Philadelphia, the reduction in the death rate from pulmonary tuberculosis has been nearly as great as that of typhoid fever.

There has been a general tendency for the death rate from pulmonary tuberculosis to decline, but it should be emphasized that the



average reduction in the control cities is as marked as in any of the cities which have improved their water supply, and greatly exceeds the reduction in one or two of them.

During the periods compared, every city but New York shows an increase in the average death rate from nephritis. The latter, with an excessive rate for the first period, shows a reduction during the second period. The lowest rates are found in Boston, Columbus, and Pittsburgh, while Cincinnati and Philadelphia show a high average rate which is slightly on the increase.

The death rates from heart disease show an increase in every case but New York, the greatest increase having occurred in Cincinnati.

The data for Chicago require separate consideration, because of differences in classification of one or two diseases, due to the fact that the statistics have been obtained from different sources.

Chicago shows a greater reduction in total death rate than any of the other cities because of the longer period involved in the calculations, and yet the reduction in typhoid mortality is no more striking than in Philadelphia, and much less than in Pittsburgh, while both Cincinnati and Pittsburgh have made greater reductions. Diarrhea and enteritis includes all ages for Chicago, and this accounts for the difference in rates. For all forms of pneumonia, Chicago shows the largest increase, which is almost equal to the reduction occurring in New York. Tuberculosis shows the greatest decrease of any of the cities considered because of the longer periods and because all forms of tuberculosis are included. The average death rate from heart disease shows a greater increase than in any of the other cities. Differences in classification enter here in addition to the factors already mentioned. The same is true of nephritis with the exception of the question of classification.

Thus far, the data show that in those cities which have made a sudden improvement in their water supplies, the only change in the death rate from any cause which can be attributed directly to the change in water supply is found in the typhoid fever rate. Among the other causes of death studied, changes equally marked, have occurred in control cities.

#### THE SEQUELAE OF TYPHOID FEVER

A study of the curves of mortality shows that in Chicago, the years of highest typhoid incidence were 1891 and 1892. During the years 1890-1894, the typhoid fever curve follows closely that of pneumonia,

the point of highest mortality occurring the same year in both. Beyond that, the peaks correspond rather closely, the pneumonia peak of 1905 coming one year after a similar typhoid peak.

Following the high typhoid rate of 1885, there is a gradual rise in the death rate from all forms of tuberculosis which continues until 1889, the greatest rise occurring in 1886. However, the curves for pneumonia, heart disease and nephritis show almost identical courses for the same period, in addition to which there are several infectious diseases predisposing to tuberculosis, the curves of which have not been plotted. In 1893, 2 years after the biggest typhoid year in Chicago, there is a distinct peak in the death rate from tuberculosis.

The curve of heart disease shows a gradual rise with irregular peaks, which is permanent and seems to bear no relation to the typhoid curve. The year 1893 shows a distinct peak in the curve of mortality from nephritis which is 2 years after the year of greatest typhoid mortality and corresponds with a similar peak in the tuberculosis curve. However, the nephritis rate shows an irregular increase up to the year 1906, after which it began to decline slowly. The curves for Chicago are of particular interest, covering as they do, a period of 32 years.

The years of epidemics of typhoid fever in Cincinnati were 1904 and 1906. There is a corresponding peak in the pneumonia curve during 1904, but a drop in 1907. Pulmonary tuberculosis shows peaks corresponding to those of typhoid fever during epidemic years, and also one in 1908, 2 years following the last typhoid peak. The rise in 1910 does not seem to bear any relation to the typhoid fever rate. Heart disease shows a jump in 1907, the year following the last typhoid peak, but in general the 2 curves show no similarity. The curve for nephritis contains peaks in the 2 high typhoid years and after the second one there are 2 additional peaks, 3 and 6 years later.

In Columbus, the years of epidemic typhoid were 1904 and 1908. There is a peak in the pneumonia curve in 1903, the year before the first high typhoid year, and one in 1910, 2 years after the second. Pulmonary tuberculosis shows a high rate during the same year as the first typhoid peak, and a similar one 2 years later. In 1910, 2 years after the second high typhoid year there is a third rise in the death rate from pulmonary tuberculosis. The maximum of 2 periods of increase in the death rate from heart disease is reached in the year following each of the years of high typhoid mortality, but these began the year before in both cases. Nephritis shows a peak in the years pre-

ceding each of the 2 high typhoid years. There is a third peak in 1913, 5 years after the last epidemic year.

The 2 years of highest typhoid mortality in Philadelphia were 1903 and 1906. No points of similarity appear between the typhoid curve and the curve of pneumonia. There is a peak in 1910, 4 years after the second high typhoid year. Pulmonary tuberculosis shows a peak in the year following the first typhoid peak and one the year after the second. The curve of mortality from heart disease shows a sharp rise in each of the years following the 2 years of high typhoid mortality. There are 3 distinct peaks in the nephritis curve, one each in 1907, 1910, and 1913. These are 1, 4, and 7 years after the second point of high typhoid mortality.

Pittsburgh, before filtration, had an excessively high typhoid fever rate, with no distinctly epidemic years. There are no points of similarity or relationship between the typhoid and pneumonia curves. The death rate from pulmonary tuberculosis begins a sharp decline the year before the fall of the typhoid curve. The curve for heart disease rises somewhat irregularly, more so after filtration of the water supply was begun, but bears no relation to the typhoid curve. The same is true of nephritis.

Charts 1 and 3 show that the mortality from typhoid fever has little influence on the total death rates, since the outlines of the curves on both charts are alike. Were this not so, the control cities might be expected to show similar curves of total death rates and death rates minus typhoid fever, while the cities with polluted water supplies ought to show curves more nearly resembling those of typhoid fever before filtration.

Diarrhea and enteritis under 2 years, the only water-borne disease in addition to typhoid fever which has been charted, shows that in Boston and New York there is no similarity between the 2 curves. In Cincinnati, however, the curves have practically the same outline with corresponding years of high incidence. There is only one corresponding peak in the curves of typhoid fever and diarrhea and enteritis in Columbus, and the sharpest rise, in 1910, occurs in a low typhoid year. Diarrhea and enteritis show only 1 peak corresponding to a high typhoid year, in 1906. The high rate of 1910 finds no corresponding high rate in typhoid fever. The curve for diarrhea and enteritis in Pittsburgh shows that the years 1903, 1906, and 1910 were years of high death rates. The first 2 are also years of high typhoid mortality,

while the last is not. The year 1910 shows a high mortality from diarrhea and enteritis in children in all the cities for which curves have been plotted, which makes it appear that some common factor, other than polluted water is involved, since during that year all the cities under consideration were supplied with unpolluted water.

The curves of mortality for Chicago require separate consideration, because of the longer periods studied and because of differences in classification. Here as well as in the other cities, the total death rate minus the typhoid component shows that typhoid mortality does not influence the course of the general mortality to any appreciable extent. The year 1891, which was a year of high typhoid mortality, shows high rates from diarrheal diseases, pneumonia and total tuberculosis in addition to an exceedingly high typhoid mortality. What part the sewage polluted water supply played in this high mortality other than in the case of typhoid and diarrheal diseases it is impossible to say without a consideration of all the factors involved. The years of greatest typhoid mortality in Chicago were 1890, 1891, and 1892. Diarrheal diseases show high rates in 1891, 1893, and 1894, in the production of which impure water supply probably played a considerable part. During the high typhoid years there are correspondingly high rates for pneumonia and all forms of tuberculosis. The latter showing an additional peak in 1893, the year after the last high typhoid year, while pneumonia has a marked rise during the next year.

Heart disease and nephritis show a gradual rise in mortality from the beginning without demonstrable relation to the typhoid mortality. Since 1907, nephritis presents a curve resembling that of typhoid fever, which is declining more rapidly than the latter.

#### GENERAL DISCUSSION

There are 2 factors which determine the ratio between the reduction in total mortality and the reduction in typhoid fever mortality. One is the rate at which the general mortality is being lowered, and the other is the height of the typhoid fever rate before filtration and the proportion due to water-borne infection. The curves of total death rate and total death rate minus typhoid component show that typhoid mortality does not greatly influence the course of the total death rate. The latter, especially in large cities, may show fluctuations quite independently of water supply, and being the resultant of a multiplicity of components, it is extremely difficult to establish its relation to a polluted



water supply. The death rate from typhoid fever is a factor which varies with each individual city and is also one of the components of the general death rate, although not a numerically important one as the curves show.

Although water is the most important source of infection in typhoid fever and the water-borne diseases generally, other articles of diet and carriers, especially, are also concerned in their dissemination. An improvement in the milk supply may lead to a decrease in the death rates from typhoid fever and other intestinal infections quite as striking as that following a sudden change in water supply. That the incidence of diarrhea and enteritis is excessively high in a city having a polluted water supply, there can be no doubt, for at least some of the infectious diarrheas in children are water-borne. Exactly what effect purification of a water supply has on the death rate from this class of diseases is difficult to determine, even by a detailed study in each instance. The proportion of diarrhea and enteritis in children due to infection is still a matter of discussion, and among the infectious diarrheas several organisms have been held to be the cause. Typhoid fever in infants is probably more common than is generally believed. In a recent study of typhoid mortality and morbidity in Pennsylvania during 1910, 1911, and 1912, Freeman<sup>22</sup> found 1623 cases and 194 deaths in children under 5 years. Without doubt, many more unrecognized cases occurred. In looking over the literature, one is struck by the fact that, about 25 years ago, cases of typhoid fever in infants were rarely recognized. Within the last few years the number of cases reported has markedly increased. A part of the reduction in diarrheal diseases is no doubt due to the disappearance of wrongly diagnosed typhoid cases.

Other factors may be of equal importance in bringing about a lowered mortality, aside from purification of the water supply. It is obvious that coincident improvement in both water and milk supplies would result in greater reduction in mortality than either alone. It would just as obviously be wrong to attribute the total effect to improvement in water supply. Landis<sup>23</sup> on the basis of his experience in Cincinnati before and after efficient milk inspection, as compared with filtration of water supply, believes that in communities which effectively purified their milk supplies the reduction in the general death rate is practically equal to that following a change from a polluted to a pure water supply. It may even be greater, since, in addition to being

<sup>22</sup> Pub. Health Rep., 1916, 31, p. 3356.

<sup>23</sup> Cincinnati Department of Health, Annual Report, 1915.



a vehicle for the transmission of the water-borne diseases, milk may serve as the medium of spreading certain other diseases through the agency of human carriers (diphtheria, scarlet fever, septic sore throat). If the drop in rates following water purification be ascribed to this change, it is just as logical to attribute any increases to the same cause.

Viable tubercle bacilli have been found in sewage-polluted streams. The extent to which tuberculosis of the intestinal tract and bone and joint tuberculosis are due to infected water is probably small. Of greater importance is the use of milk from tuberculous cows without proper pasteurization.

All public health activities are tending to lower the general death rate and lengthen the average duration of life. This results in an increase in the number of people of an age when pneumonia and the cardiorenal diseases become natural causes of death. In other words, some of those saved from an early death of infectious disease later die of the degenerative diseases. This is, therefore, one of the factors tending to increase the mortality from these diseases. To this may be added the number of deaths occurring as a sequel to typhoid fever. Among the factors tending to decrease their mortality are the education of the masses in the importance of mouth hygiene and focal infection, and a smaller number of people suffering from typhoid fever, because of improvement in water supplies and other public health activities. The relative importance of the latter is difficult to determine, but it is probably of less influence than the others mentioned. The numerous factors involved obscure the results of any study of this point.

The effect of changes in classification of causes of death upon mortality statistics is very well illustrated in the case of heart disease. "Organic diseases of the heart" of the second revision of the International List of Causes of Death includes "heart disease" and "acute endocarditis" of the first revision. The discussions in the reports of the Bureau of the Census warn against comparing statistics of heart disease before and after the second revision without taking into account the differences due to the change in classification. Yet such figures have been used to prove an undue rise in mortality from this cause since 1910.

It has been suggested that the use of a pure water supply leads to an increased resistance to infection. The facts that bacteria are constantly ingested with food, and that the intestinal mucosa normally contains a rich flora, would seem to indicate that the intestinal mucosa

has developed a high natural resistance against the ordinary organisms. The specific bacteria producing intestinal lesions are presumably absent from a pure water supply, and this is probably the most important result of purification. On the other hand, there develops among the users of an impure water supply a proportion of individuals who are resistant to water-borne infection. Those accustomed to the use of a pure water supply often become easily infected when in a community supplied with polluted water. Whether the great majority of the population uses water more freely after it has been purified is doubtful. With the exception of fondness for liquid foods and soft drinks, water intake is under the control of a reflex physiological mechanism and is not subjectively influenced.

It is not intended to minimize the importance to a community of constantly maintaining a high degree of purity in its water supply. The number of lives saved from typhoid fever alone, without consideration of the loss of time and cost of medical attention during illness and the effects of the possible sequelae of typhoid fever, would more than justify any expenditure to bring this about. I wish merely to emphasize the importance of other factors which may influence the reduction of mortality, general and specific, following improvement in water supply.

#### SUMMARY AND CONCLUSIONS

In an analysis of the mortality statistics of several cities which had made a sudden and radical change from a polluted to a pure water supply, over a period before and after improvement, no striking evidence has been found that the Mills-Reincke phenomenon and Hazen's theorem apply to these cities, when compared with control cities. The figures indicate a great variation among the cities studied, including the controls. There is, therefore, a lack of uniformity in the appearance of the Mills-Reincke phenomenon and Hazen's theorem, since some of the cities thus far studied apparently show an increase, while others show a reduction in general death rates following improvement in water supply. This is to be expected because of the numerous factors affecting the general and specific death rates.

It is extremely difficult, if not impossible, to determine the exact relation between a reduction in typhoid fever mortality and the reduction in mortality from other diseases. In the case of malarial fever, Table 5 and Chart 9 show that during high typhoid years in those cities having an impure water supply, there is in general a rise in mortality

from malaria. Following improvement in the water supply, the mortality from malaria falls to a minimum. Inaccuracy of diagnosis probably accounts for this change.

Clinical evidence indicates that pulmonary tuberculosis, pneumonia, and cardiorenal diseases may follow or complicate typhoid fever as well as any of the other infectious diseases, but a study of the mortality statistics fails to bring to light any effect of a high typhoid death rate on the death rates from these diseases. It is difficult to follow 1000 recovered cases of typhoid fever and be sure that any of the sequelae developing in later years are due to the attack of typhoid fever or to some other cause. It is infinitely more difficult to trace the direct effect of the typhoid fever death rate upon the death rates from the sequelae of typhoid fever in large cities. Even if it be assumed that tuberculosis, pneumonia, nephritis or heart disease follows in 5% of the recovered cases of typhoid fever, it would still be necessary to consider the effects of all the other infections, some of which are much greater than those of typhoid fever, before any conclusions could be drawn as to cause and effect.

The total death rate shows a tendency to decline in all the cities studied. In the control cities, the average reduction during the second period has been more marked than in some of the cities which improved their water supplies. Thus, Boston shows a greater average reduction than Columbus; while New York shows a greater average reduction than any of the other cities with which it is comparable.

The death rates from pulmonary tuberculosis also show a general tendency to decline. This tendency is more marked in the control cities than in most of the cities which have improved their water supplies. The percentage of tuberculous infection due to sewage-polluted water can at best be only very small, and its disappearance from the death rates would not produce a marked change.

The death rates from pneumonia are quite irregular, but with a general downward tendency, except in Pittsburgh. This is equally marked in the control cities.

The death rates from nephritis show a gradual rise, except in one of the control cities, New York. No changes appear which are unique for the cities with improved water supplies. The curve for heart disease also shows a decline in New York, while in the other cities there is a rise.

Considering the diseases that are sequelae of typhoid fever, as a whole, there is a general tendency toward an increase, but this is not peculiar to the cities which have had excessive typhoid fever rates.

There can be no doubt that an impure water supply may be and, in general, is accompanied by a high general death rate. Because of the multiplicity of factors involved, it is not possible to determine the exact relation between the two by a study of the general mortality statistics. Some cities will of necessity show a decrease, while others may even exhibit an apparent increase in mortality.

That a high typhoid incidence tends to increase the number of deaths from the sequelae of typhoid fever must also be true. But the large number of other factors tending to produce the same effect makes the problem very complicated, and one that probably cannot be solved by any comparison of mortality statistics.

The use of suitable controls is indispensable in studying special phases of the mortality of large communities.

# EFFECT OF THE INJECTION OF NONSPECIFIC FOREIGN SUBSTANCES ON THE COURSE OF EXPERIMENTAL RABIES \*

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Investigations on the treatment of infectious diseases have recently received additional stimuli as a result of many favorable reports on the effect of nonspecific biochemic therapy in some of these diseases. Principal among the latter are rheumatic arthritis, gonorrheal arthritis, and typhoid fever. Experimentally, it has long been known that the resistance of animals to some infections could occasionally be raised by the injection of heterogeneous nonspecific substances. Thus Pfeiffer<sup>1</sup> found that by injecting broth intraperitoneally the resistance of guinea-pigs to cholera infection was materially raised. Thus, also Babes,<sup>2</sup> Fermi,<sup>3</sup> and Repetto<sup>4</sup> claimed to have produced immunity to rabies by subsequent treatments with normal brain tissue. Fermi<sup>5</sup> claims, furthermore, to have rendered animals immune to experimental rabies by injections of fresh egg-yolk, mixtures of cholesterin and lecithin, and other lipoidal substances. The work of Babes, Fermi and Repetto have, however, not met with universal confirmation by other investigators. The objection most frequently raised to this work has been that the rabies inoculations were made subcutaneously. Their protocols present recoveries in 50-66% in relatively large series of animals. According to Koch,<sup>6</sup> however, inoculations subcutaneously of rabies virus result in positive takes in only about 50% of the animals inoculated, whereas subdural or intracerebral inoculations are positive in 100%. Repetto contends, however, that the percentage of positive infections in the subcutaneous inoculation of the virus in rats is greater than this, and that the results not in accord with his were obtained by others who did not repeat all the conditions of his experiments. The control series of

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<sup>1</sup> Hyg. Rundschau, 1900, 10, p. 357.

<sup>2</sup> Cited in Kolle and Wassermann, Handb. d. path. Mikroorg., 1913, 8, 902.

<sup>3</sup> Centralbl. f. Bakteriolog., I, O, 1907, 44, 475.

<sup>4</sup> Ibid., 1909, 51, 581.

<sup>5</sup> Ibid., 1908, 48, 357.

<sup>6</sup> Cited in Kolle and Wassermann, Handb. d. path. Mikroorg., 1913, 8, 821.



Fermi's experiments show a high mortality. He frequently worked with an equal number of animals in the control series, and the death rate from rabies invariably was 100%. His findings, that nonspecific therapy in his street virus experiments resulted in a greater number of cures than did specific treatment administered according to the routine method of Pasteur, are almost paradoxical. If legitimate criticism is to be avoided in future experimental therapeutic investigations of rabies, all animals should receive their virus inoculations subdurally or intracerebrally. True, such inoculations are most radical and do not at all

TABLE I  
RESULTS OF INOCULATION OF RABBITS WITH FRESH, FIXED VIRUS

Treatment	Day						
	1	2	3	4	5	6	7
Control.....	.....	.....	.....	.....	.....	.....	.....
Control.....	.....	.....	.....	.....	.....	.....	.....
Control.....	.....	.....	.....	.....	.....	.....	.....
Control.....	.....	.....	.....	.....	.....	.....	.....
Horse Serum.....	.....	.....	1.5 c.c.	.....	1.5 c.c.	.....	.....
Horse Serum.....	.....	.....	1.5 c.c.	.....	1.5 c.c.	.....	.....
Horse Serum.....	.....	.....	1.5 c.c.	.....	1.5 c.c.	.....	.....
Egg-White.....	1 c.c.	.....	1.5 c.c.	.....	2 c.c.	.....	.....
Egg-White.....	1 c.c.	.....	1.5 c.c.	.....	2 c.c.	.....	.....
Egg-White.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....
Egg-Yolk.....	.....	.....	.....	.....	.....	.....	.....

R. In. = rabies inoculation; ER = early symptoms of rabies; R = well developed paralytic rabies; AS = anaphylactic shock; X = death; Fat Emb. = fat embolism.

resemble the manner in which man or animals usually become infected. However, unless a therapeutic measure can be discovered which will withstand the most rigid conditions, it would scarcely seem advisable to substitute it clinically for the practically universal Pasteur method of treatment.

The work now reported covers several series of rabbits, 63 in all, 17 being controls. A portion of the work, that concerned with the treatment of fixed virus rabies, was undertaken not quite 2 years ago (Table 1). Because of the negative results, the work was discontinued

TABLE I—Continued  
RESULTS OF INOCULATION OF RABBITS WITH FRESH, FIXED VIRUS

Day									
8	9	10	11	12	13	14	15	16	17
.....	.....	.....	R. In.	.....	.....	.....	ER	R	X
.....	.....	.....	R. In.	.....	.....	.....	ER	R	X
.....	.....	.....	R. In.	.....	.....	.....	ER	R	X
.....	.....	.....	R. In.	.....	.....	.....	ER	X	
5 c.c. AS	1 c.c.	0.5 c.c.	R. In. 1 c.c.	1 c.c.	0.5 c.c.	0.5 c.c.	X		
5 c.c. AS	1.5 c.c.	1 c.c.	R. In. 1 c.c.	0.5 c.c. AS	0.5 c.c.	0.5 c.c.	1 c.c. ER	X	
5 c.c. AS	1.5 c.c.	1 c.c.	R. In. 1 c.c.	0.6 c.c. AS	0.5 c.c.	0.5 c.c.	1 c.c. ER	X	
2 c.c. AS	2 c.c.	1 c.c.	R. In. 1 c.c.	1.5 c.c. AS	5 c.c.	X			
5 c.c. AS	2 c.c.	1 c.c. AS	R. In. 1 c.c.	1.5 c.c.	0.7 c.c.	X			
5 c.c.	2 c.c.	.....	R. In. 2 c.c.	1.5 c.c.	1.7 c.c.	0.7 c.c.	ER 1.7 c.c.	X	
7 c.c.	2.5 c.c.	0.6 c.c. AS	R. In. 1 c.c.	1.7 c.c.	1 c.c. AS	1 c.c.	1.7 c.c. ER	X	
5 c.c.	2 c.c.	.....	R. In. 2 c.c.	3 c.c.	1 c.c. AS	1 c.c.	1.7 c.c. ER	X	
4.5 c.c.	2 c.c.	.....	R. In. 2 c.c.	3 c.c. AS	1 c.c.	1 c.c.	1.7 c.c. ER	X	
2 c.c.	1.5 c.c.	.....	R. In. 2 c.c.	3 c.c. AS	1.5 c.c.	1 c.c.	1.7 c.c. ER	X	
5 c.c.	2 c.c.	.....	R. In. 2 c.c.	3 c.c.	1.5 c.c.	1 c.c.	1.7 c.c. ER	X	
.....	.....	.....	R. In. 5 c.c.	4 c.c.	2 c.c.	1.5 c.c.	1.7 c.c. ER	X	
.....	.....	.....	R. In. 5 c.c.	4 c.c.	2 c.c.	1.5 c.c.	1.7 c.c. ER	X	
.....	.....	.....	R. In. 5 c.c.	4 c.c. Fat Emb.	X				

TABLE II  
RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (THREE MONTHS OLD)

Treatment	Day								
	1	2	3	4	5	6	7	8	9
Control.....	R. In.	....	....	....	....	..	....	....	....
Control.....	R. In.	....	....	....	....	..	....	....	....
Control.....	R. In.	....	....	....	....	..	....	....	....
Control.....	R. In.	....	....	....	....	..	....	....	....
Control.....	R. In.	....	....	....	....	..	....	....	....
Control.....	R. In.	....	....	....	....	..	....	....	....
Tetanus Antitoxin.....	R. In.	....	2 c.c. 300u	....	1.1 c.c.	..	....	....	1 c.c.
Tetanus Antitoxin.....	R. In.	....	2 c.c. 300u	....	2 c.c.	..	....	....	2 c.c.
Deutero-Proteose.....	R. In.	5 c.c.	....	5 c.c.	....	..	5 c.c.	....	5 c.c.
Deutero-Proteose.....	R. In.	....	5 c.c.	....	5 c.c.	..	....	5 c.c.	....
Horse Serum.....	R. In.	....	2 c.c.	....	2 c.c.	..	....	2 c.c.	....
Horse Serum.....	R. In.	2 c.c.	....	2 c.c.	....	..	2 c.c.	....	2 c.c.
Typhoid Vaccine.....	R. In.	....	1 c.c.	....	0.5 c.c.	..	....	1 c.c.	....
Typhoid Vaccine.....	R. In.	1 c.c.	....	0.5 c.c.	....	..	1 c.c.	....	0.8 c.c.
Egg-White.....	R. In.	....	2 c.c.	....	2 c.c.	..	....	3 c.c.	....
Egg-White.....	R. In.	2 c.c.	....	2 c.c.	....	..	3 c.c.	....	2 c.c.
Egg-Yolk.....	R. In.	....	2 c.c.	....	2 c.c.	..	....	2 c.c.	....
Egg-Yolk.....	R. In.	....	2 c.c.	....	2 c.c.	..	....	3 c.c.	....
Diphtheria Antitoxin..	R. In.	....	0.33 c.c. 250u	....	0.66 c.c. 500u	..	....	0.5 c.c. 900u	....
ATK.....	R. In.	1.5 mg.	....	2 mg.	....	..	4 mg.	....	5 mg.
ATK.....	R. In.	....	1.5 mg.	....	2 mg.	..	....	4 mg.	....
ATK.....	R. In.	....	1.5 mg.	....	2 mg.	..	4 mg.	....	5 mg.

R. In. = rabies inoculation; Lit. = litter of young born; ER = early symptoms of rabies; R = well developed paralytic rabies; AS = anaphylactic shock; X = death; F = fat embolism; ATK = old tuberculin Koch.

at that time. Since then, however, the successes of Miller and Lusk,<sup>7</sup> Smith,<sup>8</sup> Culver,<sup>9</sup> and others, in the treatment of certain infections have caused renewed interest, and the work has been followed by the series

<sup>7</sup> Jour. Am. Med. Assn., 1916, 66, 1756.

<sup>8</sup> Ibid., 66, 1758.

<sup>9</sup> Ibid., 1917, 68, 363.

TABLE II—Continued

RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (THREE MONTHS OLD)

Day												
10	11	12	13	14	15	16	17	18	19	20	21	22
....	....	....	....	....	....	ER	R	X				
....	....	....	....	Lit	....	....	R	R	X			
....	....	....	....	....	ER	X						
....	....	....	....	....	ER	R	R	X				
....	....	....	....	....	....	ER	R	X				
....	....	....	....	....	ER	R	X					
2 c.c.	....	3 c.c. AS	....	....	2 c.c.	....	2 c.c.	ER	R	R	R	X
2 c.c.	....	3 c.c.	....	....	2 c.c. ASX							
....	3 c.c.	....	2 c.c.	....	1.2 c.c.	R	R	R	X			
5 c.c.	....	5 c.c.	....	....	5 c.c. AS	R	R	X				
2 c.c.	....	3 c.c. AS?	....	....	2 c.c. AS	....	1.2 c.c. ER	R	R	R	X	
....	3 c.c.	....	....	2 c.c. AS	....	1.4 c.c.	ER	R	R	R	X	
1 c.c.	....	1 c.c.	....	....	1 c.c.	ER	R	R	R	R	X	
....	1 c.c.	....	....	1 c.c.	ER	R	R	X				
2 c.c.	....	2 c.c.	....	....	1.6 c.c.	....	1.2 c.c.	ER	ER	R	X	
....	3 c.c. AS?	....	....	2 c.c. ER	R	R	R	X				
2 c.c.	....	2 c.c. ASXF										
2 c.c.	....	2 c.c. AS?F?	....	....	2 c.c.	....	1.5 c.c.	....	ER	R	R	X
1 c.c. 1800u	....	1.5 c.c. 2700u	....	....	1 c.c. 1800u AS	....	0.8 c.c. 1400u	....	ER	ER	R	X
....	5 mg.	....	10 mg.	....	....	....	ER	R	R	X		
5 mg.	....	5 mg.	....	10 mg.	ER	R	R	X				
....	....	5 mg.	....	10 mg.				Lit. on 31st day				

of experiments reported herein. Rabbits weighing from 1500 to 1800 grams were inoculated intracranially and subdurally with from 3 to 5 times the minimal lethal dose of virus. They were treated by intravenous injection of horse serum, egg-white, egg yolk, broth culture-media, globulin of horse serum (both antitetanus and antidiphtheria serum being used), typhoid vaccine and tuberculin. The maximum doses of these substances compatible with the physical condition of the

animal were used. Occasionally a reaction suggestive of sensitization was obtained; this was combatted by the immediate intracardiac administration of adrenin when necessary. A few of the animals succumbed in attacks resembling typical acute anaphylaxis, and grossly, the visceral changes were suggestive of this. The animals treated with egg-yolk appeared most refractory. A considerable number, not recorded in the tabulations, died at the time of the second injection with symptoms quite characteristic of anaphylaxis, the changes in these, also, being equally suggestive of anaphylaxis. Frozen section of lung, stained with sudan III, however, revealed a pulmonary vascular system filled and plugged with sudan III-stained globules of varying sizes.

The egg-white was prepared by mixing the white of hen's eggs with an equal volume of distilled water. The mixture filtered under aseptic precautions, was injected without further dilution. The egg-yolk was similarly prepared. The deuteroproteose was prepared from milk and dissolved in physiologic salt solution. It was injected as a 1% solution. The horse serum was used undiluted in doses, as indicated in the tabulations. The serum-globulins (antidiphtheria and antitetanus) were diluted with an equal amount of physiologic salt solution; the amounts of the diluted globulin injected and their value in antitoxin units are indicated in Table 1. The tuberculin was diluted with physiologic salt solution and injected in volumes of 5 c.c. The quantity of tuberculin in milligrams received in each injection is recorded in the tables. The animals treated with broth culture-media received this in dilutions equal to that of the tuberculin. They were injected and carried along as controls for the tuberculin-treated animals when it was found in a late series that some of these animals did not

TABLE III

RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED FIXED VIRUS (SIX MONTHS OLD)

Treatment	Day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	R. In.	....	....	....	....	ER	R	R	X					
Control	R. In.	....	....	....	....	....	ER	R	R	X				
ATK	R. In.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg. ER	10 mg. R	X					
ATK	R. In.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg. ER	10 mg. R	10 mg. R	10 mg. R	X			
ATK	R. In.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	10 mg.	....	10 mg.	10 mg.	....
Broth	R. In.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	....	5 c.c.	5 c.c.	5 c.c.	....	5 c.c.	5 c.c.	....

R. In. = rabies inoculation; ER = early symptoms of rabies; R = well developed paralytic rabies; X = death; ATK = old tuberculin Koch.





TABLE IV  
RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (FOUR MONTHS OLD)

Treat- ment	Day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	R. In.	....	....	....	....	....	....	....	....	....	....	....	....	ER
Control	R. In.	....	....	....	....	....	....	....	....	....	....	....	....	....
ATK	R. In. 10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	10 mg.
ATK	R. In. 10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	10 mg.
ATK	R. In. 10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	10 mg.
ATK	R. In. 10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	10 mg.
ATK	R. In.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.
ATK	R. In.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.	.... 10 mg.	10 mg.
Broth	R. In.	5 c.c.	.... 5 c.c.	5 c.c.	.... 5 c.c.	5 c.c.	.... 5 c.c.	5 c.c.	.... 5 c.c.	5 c.c.	.... 5 c.c.	5 c.c.	.... 5 c.c.	5 c.c.

R. In. = rabies inoculation; ER = early symptoms of rabies; R = well developed paralytic rabies; X = death; ATK = old tuberculin Koch.

virus. Extended exposure to light, air, and gradual desiccation have a marked attenuating action on rabies virus. At 33⅓% neutral glycerin mixture at 4 C., protected from light rays, is the most favorable agent for preserving it. Fixed virus kept in this way has in some instances retained its virulence to a somewhat lessened degree after a period of from 3 to 3½ years. Street virus is said to retain an undiminished virulence when preserved for from 80 to 90 days under these conditions. Negri bodies are still recognizable in smears made at this time, according to Mazzei.<sup>10</sup> After preservation for longer periods, the virus diminishes in virulence gradually, slowly, but progressively. It was, therefore, decided to resume investigation with street and fixed virus that had slowly become attenuated by age, while preserved under otherwise favorable conditions. Accordingly, a street virus in which Negri bodies were present in abundance and which had never failed to infect when inoculated subdurally, was preserved in 33% neutral glycerin, in the dark, at 4 C., for 3 months. In inoculations with this virus in its fresh state, positive symptoms were observed usually as early as the 16th day, and death usually occurred on the 20th or 21st day, as illustrated by the protocols of the first 2 rabbits (Table II).

Twenty-two rabbits constituted the second series (Table II). Six were controls, the remainder, following their inoculation, were treated with serum globulin (antitetanus and antidiphtheria), deuteroproteose, horse serum, typhoid vaccine (1,000,000,000 per c.c.), egg-white, egg-yolk, and tuberculin. Three rabbits were treated with serum globulin, 2 receiving it in an antitetanus form

<sup>10</sup> Cited in Kolle and Wassermann, Handb. d. path. Mikroorg., 1913, 8, 866.

TABLE IV—Continued

RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (FOUR MONTHS OLD)

Day																
15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
R	R	R	R	R	X											
....	10 mg.	10 mg.	....	10 mg.	....	10 mg.	....	10 mg.	10 mg.	...	10 mg.	...	10 mg.	...	10 mg.	10 mg.
....	10 mg.	10 mg.	....	10 mg.	....	10 mg.	ER	X								
....	10 mg.	10 mg.	....	10 mg. ER	X											
....	10 mg.	10 mg.	....	10 mg.	....	10 mg.	....	10 mg.	10 mg.	...	10 mg.	...	10 mg.	...	10 mg.	10 mg.
10 mg.	....	10 mg. ER	10 mg. R	10 mg. R	X											
10 mg.	....	10 mg.	10 mg.	10 mg.	....	10 mg. ER	R									
						X	X									
5 c.c.	....	5 c.c. ER	5 c.c. ER	R	R	X										

and 1, antidipteria. They received from 6 to 7 intravenous injections during the first 15 to 17 days following inoculation. One succumbed on the 15th day with symptoms resembling anaphylaxis, and necropsy findings were typical. Of the remaining 2, the earliest symptoms of rabies appeared on the 18th day, and in the other, on the 19th day. Both died on the 22nd day after a typical course of paralytic rabies. Two rabbits received from 5 to 6 injections of a 1% solution of deuteroproteose during the first 15 days following their inoculation. Both developed symptoms of paralytic rabies on the 16th day; one died on the 18th day, the other on the 19th day. Two rabbits treated with horse serum received 7 intravenous injections during the 17 days following their inoculation with virus. Both developed initial paralysis on the 17th day, and after a typical course of the disease, death occurred, in both instances, on the 21st day. Two other rabbits received 6 injections of typhoid bacterin during the 15 days following their inoculation with virus. Both developed typical paralytic rabies, one on the 15th day, the other on the 16th day. The former died on the 18th day, the latter on the 21st day after the inoculation. Two additional rabbits received 6 intravenous doses of egg-white, during 15 days following their inoculation. In 1, early symptoms of rabies appeared on the 14th day, and death, on the 18th day. In the second, the earliest manifestations of rabies were present on the 18th day, whereas death occurred on the 21st. Another pair of rabbits received egg-yolk intravenously; 1 died of fat embolism on the 12th day, at the time of the 5th injection, and before symptoms of rabies had developed. The other received 7 injections during 17 days after inoculation. Typical paralytic rabies developed on the 19th day and death occurred on the 22nd day. Three rabbits received 6 intravenous injections of tuberculin during 14 days. In 1, rabies developed on the 15th day, and death occurred on the 18th day. The second

rabbit manifested its earliest symptoms on the 17th day, death occurring on the 20th day. The third rabbit, however, developed no symptoms. She appeared normal for a period of ten weeks after inoculation. During this time, she gave birth to a litter of 4 normal young. Following this, she was inoculated a second time with the same virus used in the first inoculation. She received 2 injections of tuberculin on the 2 following days, during which time she appeared perfectly normal. She was found dead on the morning of the third day. Death appeared to have been due to a localized, acute, encephalitis at the site of the second inoculation. She had, however, unquestionably withstood the first inoculation made ten weeks before. I do not believe that her pregnancy occurring during this period was a factor in her survival. It has been my experience and the literature in general indicates that pregnancy has no effect on the course of experimental rabies. This was the only rabbit of series II to survive. The course of the rabies in the other animals was not materially altered following treatment with the various substances administered. The controls all ran a typical course of the disease and died well within the 21-day limit of this particular virus strain.

While series II was in progress, a third series of 6 rabbits was inoculated with an old fixed virus, of the same strain as that used in series I. It was a virus-containing brain tissue which had been preserved in the dark for 6 months at 4 C. in a 75% neutral glycerin solution. Two of the inoculated rabbits were reserved as controls. Three were treated with tuberculin and 1 with broth, intravenously. Table I showed that this virus in its fresh state always produced the first symptoms of rabies on the 5th day after inoculation, and that death usually occurred on the 7th day. The controls inoculated with this same but age-attenuated virus, in series III, developed their first symptoms on the 6th and 7th days, respectively, and in each, death occurred on the 4th day of the paralysis.

TABLE V  
RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (FOUR AND ONE-HALF MONTHS OLD)

Treatment	Day									
	1	2	3	4	5	6	7	8	9	10
Control.....	R. In.	....	....	....	....	Ab				
Control.....	R. In.	....	....	....	....	....	....	....	....	....
Control.....	R. In.	....	....	....	....	....	....	....	....	....
Broth.....	R. In.	5 c.c.	....	5 c.c.	....	5 c.c.	....	5 c.c.	5 c.c.	....
Broth.....	R. In.	5 c.c.	....	5 c.c.	....	5 c.c.	....	5 c.c.	5 c.c.	....
ATK.....	R. In.	20 mg.	....	20 mg.	....	10 mg.	....	10 mg.	10 mg.	....
ATK.....	R. In.	20 mg.	....	20 mg.	....	10 mg.	....	10 mg.	10 mg.	....
ATK.....	R. In.	20 mg.	....	20 mg.	....	10 mg.	....	10 mg.	10 mg.	....

R. In. = rabies inoculation; ER = early symptoms of rabies; R = well developed paralytic rabies; X = death; ATK = old tuberculin Koch; Ab = abscess.

One of the tuberculin-treated animals of this series developed symptoms of rabies on the 7th day, and died on the 9th day. A second tuberculin-treated animal became paralyzed on the 8th day, and died on the 11th day. The remaining 2 rabbits, one treated with broth injection, and the other with tuberculin, were given 10 and 11 injections, respectively, during 13 days following their inoculation. During this time, they appeared normal. These animals continued apparently normal more than 3 weeks. On the 31st day following their first inoculation, they were reinoculated with the same virus, now 214 days old. The rabbit formerly treated with tuberculin again received daily treatments of the same; the rabbit previously treated with broth culture-media received the same broth treatment. The broth-treated rabbit died suddenly on the second day following its reinoculation, apparently of an acute encephalitis, at the site of its second inoculation. The tuberculin-treated rabbit appeared normal for 5 days. Late on the 6th day, there was an early paralysis which progressed, but was incomplete at the end of the 7th day. On the 9th day, paralysis was complete, and death occurred on the 10th day.

The results obtained in Series II and III are too meager to justify general conclusions. It was not apparent why those animals which had apparently survived their first inoculation should have succumbed or have been more susceptible to a subsequent reinoculation. Was the treatment which they had received following their first inoculation a therapeutic factor which had raised their resistance at that time so that they survived this inoculation? If so, it had not resulted in a perma-

TABLE V--Continued

RESULTS OF INOCULATION OF RABBITS WITH TIME-ATTENUATED STREET VIRUS (FOUR AND ONE-HALF MONTHS OLD)

Day															
11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
....	....	....	....	....	....	ER	R	R	X						
....	....	....	....	....	....	ER	R	R	R	X					
5 c.c.	....	5 c.c.	....	5 c.c.	5 c.c.	ER	5 c.c. R	R	5 c.c. R	R	X				
5 c.c.	....	5 c.c.	....	5 c.c.	5 c.c.	....	5 c.c.	....	5 c.c.	....	5 c.c.	....	5 c.c. ER	X	
10 mg.	....	10 mg.	....	10 mg.	10 mg.	....	10 mg.	....	X						
10 mg.	....	10 mg.	....	10 mg.	10 mg.	....	10 mg.	....	10 mg.	....	10 mg.	....	10 mg.	ER	X
10 mg.	....	10 mg.	....	10 mg.	10 mg.	....	10 mg. FR	R	10 mg. R	R	10 mg. R	R	10 mg. R	X	



nent or even temporary immunity. Furthermore, this same treatment had proved totally inadequate in inhibiting or preventing infection from a subsequent reinoculation with the same infecting agent. This latter condition, however, is not incompatible with our experience in some other infectious diseases. Thus, not infrequently, reinfection with syphilis subsequent to a former initial mild luetic infection, manifests itself clinically and symptomatically as a most virulent type. Another explanation, that of faulty technic at the time of the first inoculation, does not come into question. In several hundred experimental intracerebral inoculations with nonattenuated rabies virus, I have never before failed to infect the animal with rabies.

Because of the inconclusive results so far obtained, a fourth series of 9 rabbits was inoculated with the same street virus used in the series tabulated in Table II. This virus, preserved under the same conditions was now 122 days old. The results in this series are recorded in Table IV. Two of the rabbits of this series were reserved as controls; 1 was treated with broth, the remaining 6 received tuberculin. The rabbits received 4 intravenous injections of broth or tuberculin per week, as indicated in the table. The broth-treated rabbit developed early symptoms of paralytic rabies on the 17th day and succumbed on the 21st day. Four of the tuberculin-treated rabbits died of rabies. Death occurred in 3 of these, after a period of illness of only 24 hours' duration. The remaining 2 continued to receive tuberculin 4 times per week for 31 days, when it was discontinued. These rabbits remained apparently normal, and at this date, 75 days after their inoculation, are alive and apparently normal. One of the control animals developed symptoms of early paralysis on the 14th day and succumbed on the 20th day, after 5 days of complete paralysis. The remaining control animal went into convulsions immediately following the trephining and intracerebral inoculation. During the course of these convulsions it severely traumatized its right cheek by violent contact with the walls of its cage. On the 3rd day, an extensive cellulitis involving all of the soft parts of the right half of the head developed. This finally localized in a large submaxillary abscess which, when opened and curetted, was found to have its focus in a suppurative osteitis of the lower jaw. This was repeatedly opened and curetted and drained through several fistulas until January 23, at which time healing was almost complete. During this time the animal failed to manifest any symptoms of rabies and it can be said that it is well beyond any possibility of developing it. The survival of this control animal vitiated any conclusions which may have been drawn from the recoveries among the treated animals. It might have been argued that recovery of this control could have been due to the severe suppurative contemporary infection, existing during the period of incubation, following the inoculation with rabies virus. However, it raised the just doubt that we were now dealing with a virus so attenuated that it was no longer sufficiently potent to produce disease in 100% of inoculations in untreated animals.

Because of this new complication, a final series of 8 rabbits was inoculated with the same street virus, which at this time was 137 days old. Three of these animals were reserved as controls, 2 were treated with broth, and 3 received tuberculin. The results obtained in this series are recorded in Table V. The periods of paralysis of the rabbits that died were of varying durations. In one

instance, a tuberculin-treated rabbit, which at no time presented symptoms of rabies, was found dead on the morning of the 20th day. Necropsy revealed an extensive subcutaneous, pulmonary and splenic tuberculosis. Other treated rabbits succumbed within 24 hours of their first paralytic symptoms, whereas one of the tuberculin-treated animals lived a week after the earliest symptoms developed. All of the treated animals died, 1 on the 21st day, 1 on the 22nd day, 2 on the 25th day, and 1 on the 26th day. One of the controls died on the 25th day after typical paralytic rabies. The other control animal still lives, and is now apparently normal, and well beyond the possible development of rabies from the former inoculation. I had hoped, after my experience in the first few series of these experiments, that tuberculin might prove to have a beneficial effect in encephalitis due to rabies, as has some time been claimed for it for syphilis by von Yaregg<sup>11</sup> and others who have used it in extremely large doses in the treatment of the chronic encephalitis of general paresis. The scope of these experiments, extended as they are, are not extensive enough to warrant any favorable conclusions. In a desperate case of rabies in man, in which death appeared certain, and the possibility of activating a latent tuberculosis was negligible because of this apparent certainty of death from rabies, it might be justifiable to administer tuberculin in superdoses as a measure of last resort.

#### CONCLUSIONS

The injection of certain nonspecific substances (horse serum, serum globulin, egg-white, egg-yolk, broth, typhoid vaccine, or tuberculin) does not inhibit the course of experimental rabies in rabbits produced by nonattenuated virus. The seemingly beneficial effect of tuberculin in the early series of the experiments must be disregarded because of the survival of some control animals inoculated at a later date with the same virus.

The results of Fermi and Repetto cannot be obtained with egg-yolk injections when potent rabies virus is inoculated subdurally or intracerebrally in rabbits.

Rabbits surviving an intracerebral inoculation of attenuated rabies virus (fixed or street virus), may become hypersensitive to a reinoculation of the same virus made in the same way.

<sup>11</sup> Wien. med. Wchnschr., 1909, 49, p. 21.



# THE EPIDEMIOLOGY OF PELLAGRA IN NASHVILLE, TENNESSEE, II \*

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## INTRODUCTION

A report concerning the epidemiology of pellagra based on a partial survey of the city of Nashville has been previously published<sup>1</sup> in which the physical aspects of the city, the relation of pellagra to the method of sewage disposal, the diet, as well as the evidence of the occurrence of contact in the development of new cases have been fully entered into.

The present paper concludes the field work connected with this survey, which was amplified and completed during the summer of 1916. The survey included the entire city as well as the immediate adjacent suburbs. We have found it expedient to adhere closely to the program laid down for the preliminary survey, that is to cover, in as complete a manner as possible, all phases which might be of interest and to present the results from an impartial point of view, simply suggesting an interpretation of those findings which seemed clear and convincing to us.

## ORGANIZATION OF SURVEY

The work carried on during the season of 1916 has been organized under the following divisions:

1. *General Survey*.—(a) A house-to-house survey with inspection of all inhabitants similar in character to that carried on in 1915, but covering the entire city and its environs.

(b) An inspection of all school children in the schools, carried on with the cooperation of the school board and the medical inspectors of the board. All suspected cases were later examined at the homes.

\* Received for publication January 18, 1917.

<sup>1</sup> Jobling and Petersen: *Jour. Infect. Dis.*, 1916, 18, p. 501.

(c) Factory inspection covering the larger industrial establishments of the city.

(d) Inspection of the public and semipublic institutions, such as orphanages, asylums, etc.

2. *Prevention* (under direction of Dr. W. H. Hibbett, the City Health Officer).—(a) Dispensaries. Five dispensaries were maintained during the summer months with a physician in attendance afternoons and evenings.

(b) Food distribution. In cooperation with the survey and the dispensaries, the United Charities of Nashville and the Davidson County Charities effectively assisted our work through the free distribution of milk and eggs whenever it was found that a pellagrin was without sufficient funds to add such articles of diet to his ration. One quart of milk or buttermilk per day and a dozen eggs per week were furnished from this source for each indigent patient.

(c) Sanitation. Because of the chaotic political conditions during the past few years, systematic progress in sanitation has been neglected. Through the publicity attending a vigorous antityphoid campaign of the health officer and the wide-spread interest aroused in health matters through the house-to-house canvas of the city, a demand for improved sanitation has been created and is meeting response in an effort to extend the sewerage system.

#### GENERAL STATISTICS

The population of Nashville at present is 98,700, with an additional 16,500 occupying the immediate adjacent territory, making a total of 115,200. This is composed of:

White Adults .....	57,150	
White Children .....	24,150	
		81,300.....70% of total
Colored Adults .....	22,700	
Colored Children .....	11,200	
		33,900.....30% of total

During the surveys of 1915-1916, we have obtained the names of 1664 pellagra patients who have lived in the survey area in recent years. Of this number, 75% were whites and 25% colored. It is possible that the incidence is equal for the 2 races; the slightly higher proportionate ratio for the whites is likely due to the fact that the possi-



bilities for diagnosis and attention among the whites is greater, so that more cases have come to our attention.

Of the total number of cases, some 535 have died; of the remaining number, we have obtained accurate data for 930, which form the basis for the following study.

*Age Incidence.*—Our results for the total number of cases corresponds in general to the figures obtained during the previous year, with the exception that the period of childhood (up to 16 years) has been found to yield relatively more cases than observed last year. This is, no doubt, due to complete examination of all school children directly in the schools; under less favorable conditions, children were apt to escape observation. When the duration of life is divided into 3 periods, as is followed in the various charts: childhood (to 16 years), adults (16-50 years), old age (over 51 years), the percentage of cases is as indicated in Table 1.

TABLE 1  
PERCENTAGE OF PELLAGRA ACCORDING TO PERIOD OF LIFE

Period	Whites		Colored	
	Males %	Females %	Males %	Females %
Childhood.....	57	30	82	37
Adults.....	16.3	51.5	9	44
Old age.....	26.7	18.5	19	19

The proportions apply in general to both races, that is, among the males, the greatest incidence occurs in childhood, a diminution, in adult life, and an increase, with advancing age; among the females, on the other hand, the greatest incidence occurs during adult life, with a relative decrease in advanced life.

The percentage incidence for 5-year periods is illustrated in Chart 1.

*Sex Incidence.*—Of the total number of white cases, 42% occurred in males and 58% in females; among the colored, 40% occurred in males and 60% in females.

*Year of Onset.*—During the present season (1916), pellagra has been less prevalent and decidedly milder in its manifestations than during the preceding 2 years; our observations correspond with those made elsewhere in the South in this respect. On observing the year of onset (Chart 2), the impression is gained that a larger number of cases originated during the present season, especially among the males.

This is due solely to the fact that in obtaining the histories of pellagrous children, a large number denied having had lesions previous to the time of inspection, although we have every reason to believe that the lesions had been present for 1 or more years and had been overlooked or disregarded by the parents. The characteristically mild character of pellagra in children makes this very probable.

*Season of Onset.*—The majority of our patients have stated that their 1st lesion commenced in summer, the months of June and July being apparently the period of greatest activity. As noted in our previous report, the colored race is seemingly somewhat more resistant in its relation to the seasonal onset, only 20% occurring in the spring, as compared to 29% for the white (Chart 3).

*Character of Lesions.*—In both races, the skin lesion was apparently the most obvious symptom brought to the attention of the patient, less than one-fourth of the patients referring their first symptoms to either the gastro-intestinal or nervous symptom. Among the females, however, a large number gave the gastro-intestinal involvement as the dominating symptom. It is to be remembered, however, that mild and transitory gastro-intestinal symptoms are of more frequent occurrence early in the course of the disease than would be inferred from Chart 4. Indeed, a history of stomatitis will often be obtained on careful questioning, where other symptoms have not yet made their appearance.

*Condition of Patient When First Examined.*—We have endeavored to group the patients found in these surveys into 2 classes, one with active symptoms: acute skin changes, persistent diarrhea, stomatitis, active mental changes; and one with chronic symptoms, in which the characteristic atrophy of the skin of the hands or feet, with occasional diarrhea, or in which evidence of degenerative changes in the central nervous system are apparent, cases in which the disease process is more or less quiescent. In a certain number of cases this information was not clearly obtained. The results are given in Table 2.

TABLE 2  
PERCENTAGE OF PELLAGRA ACCORDING TO SYMPTOMS

Symptoms	Whites		Colored	
	Males %	Females %	Males %	Females %
Active lesions.....	42	33	56	45.5
Chronic lesions.....	47	60	36	54
Undetermined.....	11	7	8	

These figures substantiate in a general way the impression that in the colored race pellagra is apt to show greater activity in its manifestations and the course of the disease is without doubt shorter than among the whites.

#### DOMICILE

*Number of Rooms.*—Of the white cases, 2% lived in single room houses, 20% in 2-3 room houses, the balance in houses containing more than 3 rooms. Of the colored cases, 1.2% lived in single room houses, 35% in 2-3 room houses, the balance in houses containing more than 3 rooms.

*Number of Inhabitants in House.*—The number of cases in which a single person inhabited a house and became ill was small, being slightly more than 1% for the white and 4.5% for the colored; with only 2 exceptions, these were all females. In 24% of the white cases, 2 or 3 persons were living in the house at the time the pellagrin developed symptoms, and in 70%, 4 or more were living in the house.

The corresponding figures for the colored were 25% (2-3 persons), and 68% (4 or more persons).

*Screening.*—Screening was present in 16% of the houses in which the patients were living at the time they developed the disease. In the great majority of instances, however, the screening was full of holes, and the houses contained many flies and mosquitoes. About 15% of the cases developing in these houses were secondary to others belonging to the same family.

*Bathing Facilities.*—As a relative index of the hygienic demands and economic status of the individual, the presence of bathrooms in the houses was ascertained. Among the white cases, 5% were so equipped, while among the colored, only 1 case in the 251 commanded such facilities. Contrary to the general impression, however, the colored managed to maintain a fair state of personal cleanliness.

*Domestic Animals.*—Thirty per cent. of the white cases maintained domestic animals of some variety (cats, dogs, fowl, horses, and swine) about the premises. Among the colored, approximately the same proportion holds true, that is, 32%.

*Rodents.*—Seventy-nine per cent. of all the white houses were infested with mice or rats, the latter predominating. Among the colored, their presence was ascertained in all cases.

*Insects.*—Despite screening in 16% of the houses, we found only 2% in which flies were not present. Practically all houses abounded in

mosquitoes, roaches, and fleas; a large number admitted the presence of the *Cimex lectularius*. We are not able, however, to give the exact percentage harboring these insects, nor the number infested with body parasites.

*General Cleanliness.*—The cleanliness, or its lack, of the family and the home was noted by the examiners under 3 heads: good, fair, and bad. The percentage for the 2 races was as follows:

	Whites	Colored
Good.....	24%	10.8%
Fair.....	44%	36 %
Bad.....	22%	40 %
Undetermined.....	10%	Undetermined.. 13.2%

#### ASSOCIATED FACTORS

*Poverty.*—The economic factors will be later fully entered into; we wish, however, to present briefly here the number of cases by whom charity was accepted. It should be understood that large numbers of these cases were not paupers in the accepted sense of the term, but pellagrins who, because of the relatively high cost of milk and eggs felt that they could not afford the addition of these elements to their daily ration and were therefore willing to accept the assistance rendered by the United Charities. Of the total number of white cases, 16% accepted such assistance, and 26% of the colored cases.

*General Physical Conditions of the Family.*—The examiners, whenever possible, stated in the history of their cases the impression of the physical condition and approximate state of nutrition apparent to causal observation of the members of the family. These were classified as good, fair, and bad. The relative percentages are as follows:

	Whites	Colored
Good.....	35%	32%
Fair.....	40%	43%
Bad.....	15%	16%
Undetermined.....	10%	Undetermined.. 9%

*Associated Diseases.*—Tuberculosis, syphilis, and intestinal parasites are recognized as commonly associated with pellagra. In our cases, it was, of course, impossible to make a complete physical examination; therefore, only the frank cases of these diseases could be ascertained. The percentage of tuberculosis, syphilis, and hookworm disease among the white and colored is seen in Table 3.

TABLE 3  
PERCENTAGE OF TUBERCULOSIS, SYPHILIS, AND HOOKWORM DISEASE

Disease	Whites		Colored	
	Males %	Females %	Males %	Females %
Tuberculosis.....	1.4	5	0	4
Syphilis.....	1	0.8	6	1.4
Hookworm disease.....	0	0	0	0.33

#### ECONOMIC CONDITIONS

We have endeavored to make a most accurate study of the economic condition of pellagrous patients. In order to do this, our examiners ascertained the average rentals for the entire city, the weekly income of the pellagrin when a wage earner, and the total income of the pellagrous family; from this latter data, we have computed the average amount of money available for each pellagrin per week. (The total family income divided by the number of individuals, in which computation children have been accorded the same value as adults.)

*Demand for Labor.*—In order to obtain an approximate idea of the economic conditions prevalent during the past 2 years, we have obtained the average number of employees in several representative industries, including textile, lumber, and iron factories. The figures for these establishments for the period 1912-1916 are as follows:

1912.....	3043
1913.....	3032
1914.....	3101
1915.....	3085
1916.....	3116

As far as these leading industries are representative, there was no marked fluctuation in the number of employees during the years recorded. The wage scale was not altered during the time under consideration.

*Average Wage per Week.*—Of the white adult male pellagrins, 86, or 70%, were wage earners, of whom more than 60% earned 10 dollars per week or more. Of the adult white females, 62, or 22%, were wage earners, and of these, 56% earned less than 10 dollars per week (Chart 5). Of the colored adults, 64% of the males and 38% of the females were wage earners. Of the males, 66% earned less than



10 dollars per week, while of the females a similar percentage earned less than 8 dollars per week.

In a comparison of the relative economic condition of the white and colored as indicated by the wages, certain local customs must be considered. The colored females work largely as cooks and laundresses, and usage demands, especially in the former occupation, that the employer supply the cook with sufficient food to feed her family. In this way the actual emolument is greater than would be indicated from these figures. It is of course not possible to state in just how great a measure this factor modifies the conditions.

*Average Income per Individual per Week.*—When we have estimated the amount of money available for each pellagrin per week (data obtained from 580 white out of a total of 679), the percentage illustrated in Chart 6 is obtained. Of the whites, more than half (56.5%) have an available income of 2 dollars and a half per week and over. Apparently only one-fourth (24%) of the colored pellagrins have an income of this amount.

Among the differences in the economic status of the 2 races are found: 1. The rental outlay of the average colored family is 4-6 dollars less per month than for the average white family. 2. The addition of food and clothing in part payments for services of the colored not credited in the money income. 3. The probability of a greater per capita consumption of alcohol among the whites. These 3 factors would tend to equalize to a certain extent the difference in income, although we do not believe them sufficient to change the unfavorable balance for the colored race.

*Rentals.*—The rentals form a fairly reliable basis in estimating the economic status of the pellagrous class. The percentages are graphically illustrated in Chart 7.

Of the whites, 11% either owned their own homes, or were buying them on the installment plan, indicating a certain amount of thrift. Sixteen per cent. of the colored pellagrins also owned their homes, or were buying them. The rentals paid by the balance are practically all under 15 dollars per month, only 3% of the cases occurring in families paying more than this amount. Of the colored families, few pay more than 8 dollars per month. The relation of the origin and distribution of both white and colored cases to the rentals for the entire city can be observed by reference to the appropriate overlay maps accompanying this report.



- UNIMPAVED
- REPAVED SURF ROAD
- 6-10-15
- 15-20
- \$200-400
- R \$40-

Map of [illegible] showing [illegible] and [illegible] of the [illegible] [illegible] [illegible]







- UNINHABITED
- RENT UNDER \$5.00 per Month
- \$5.00 - 10.00
- \$10.00 - 20.00
- \$20.00 - 40.00
- OVER \$40.00

Map 1. Relation of the origin and deaths from pellagra to the economic condition (rental value)







Map 1. Relation of the lake and south from village to the sea. (Note: Some points represent area supplied by water cottage sewerage system.)





Map 2.—Relation of the origin and death from pellagra to the sewerage system. Shaded portions represent area supplied by water carriage sewerage system.







- Federal Land
- State Land
- Private Land
- Indian Land
- National Forest
- Bureau of Reclamation
- Bureau of Fish and Game
- Bureau of Land Management

Map of Klamath River watershed  
showing land ownership and  
management.







Map 3. Relation of the origin and death from pellagra to the density of population.



*Occupation.*—Associated with the economic question is that of employment. The occupations engaged in and percentages for males and females, whites and colored, are represented in Table 4.

TABLE 4

PERCENTAGE OF MALES AND FEMALES, WHITES AND COLORED, ENGAGED IN VARIOUS OCCUPATIONS

Occupation	Whites		Colored	
	Males %	Females %	Males %	Females %
School.....	41	17	45	27
Common labor..	16		12	
Housework.....		60		35
Lumber.....	11	0.75		
Iron.....	0.75		2	
Fertilizer.....	0.35			
Textile.....	1	3		
Mercantile.....	2	0.5		0.66
Professional.....	0.35	0.5	3	1.33
Farming.....	3.5		2	
Laundry.....		1.5		20
Miscellaneous...	2.1	0.25		
	78.35	88.5	64	84

Apart from common labor, it is interesting to observe a relatively high percentage of men occupied in the lumber industry. This branch, one of the most extensive in Nashville, offers fairly reasonable remuneration. Among the female cases, only 3% were employed in the textile mills.

#### SANITATION

*Sewerage.*—In our previous report, we discussed the relation between the development of pellagra and the method of sewage disposal. The greater part of that survey was made in the poorer portion of the city, where pellagra was known to occur most frequently. The survey of this season covered the entire city, and some of the immediately adjoining districts.

Of the total white population of the city, 60% live in sewered houses, the balance have only the surface privy. For the colored population, the proportions are reversed, 66% living in houses without

sewerage connection and only 34% in houses with access to sewerage facilities (Chart 8).

For the pellagrins, we found that the figures obtained last year have been maintained. Of the white cases, only 16% originated in houses with sewer connections, and of these, more than half were in houses provided with the so-called alley sewer, a water privy in an outhouse at the rear of the domicile, most frequently in an un-sanitary condition. For the colored, the same general proportion holds true, with the exception that the water privies were practically all of the alley-sewer type.

*Water Supply.*—There is apparently no connection between the water supply and the development of pellagra. The percentage of pellagrins using the various water supplies is seen in Table 5.

TABLE 5  
PERCENTAGE OF PELLAGRINS USING VARIOUS WATER SUPPLIES

Water Supply	Whites %	Colored %
City water. . . . .	50	55
Well-pump.....	11	6
Well-bucket.....	25	18
Spring....	2.5	7
Cistern.....	7	10

#### DIET

*Cost of Food.*—In describing the economic condition prevalent in Nashville for the past few years, we have endeavored to bring out the food purchasing power of the people; inasmuch as this is influenced by the cost of food-stuffs, we shall present, first, the average wholesale price of a variety of staple foodstuffs current in Nashville since 1910. The averages for the 2 yearly periods (1st and 2nd halves of the year) are given in Table 6.

It will be observed that the food cost remained practically stationary from 1910 until the summer of 1916, since which time the figures have, with only a few exceptions, almost doubled; these increases holding true not only for the protein, but also for the carbohydrate and fat elements.

*Deficiency in Quantity Consumed.*—The quantity of food consumed in order to satisfy the appetite of the individual is decidedly variable,



TABLE 6  
AVERAGE WHOLESALE PRICE OF FOODSTUFFS CURRENT IN NASHVILLE SINCE 1910

Year	Period	Flour	Meat	Beans	Corn Meal	Butter	Lard	Potatoes	Rice	Sugar	Molasses
1910	1st half	\$5.05	\$0.15	\$2.66	\$0.31	\$0.20	\$0.11	\$2.56	\$0.06	\$4.92	\$0.31
	2nd half	4.70	0.158	2.75	0.30		0.11	2.50	0.061	5.70	0.30
1911	1st half	5.00	0.135	2.57	0.30	\$0.20-0.35	0.10	2.00	0.055	4.93	0.30
	2nd half	4.70	0.155	2.65	0.29		0.11	2.50	0.055	5.10	0.30
1912	1st half	5.00	0.13	2.67	0.30	0.20-0.25	0.10	2.58	0.06	5.00	0.31
	2nd half	4.70	0.16	2.79	0.29		0.11	2.50	0.06	5.10	0.305
1913	1st half	5.00	0.14	2.60	0.30	0.20-0.40	0.10	2.66	0.06	4.94	0.29
	2nd half	4.60	0.16	2.62	0.28		0.11	2.51	0.06	5.06	0.31
1914	1st half	5.00	0.14	2.60	0.30	0.20-0.40	0.12	2.60	0.065	4.60	0.295
	2nd half	4.70	0.16	2.56	0.30		0.12	2.56	0.065	5.90	0.30
1915	1st half	4.90	0.145	2.60	0.31		0.12	2.60	0.062		
	2nd half	4.50	0.16	2.60	0.30		0.11	2.62	0.062		
1916	1st half	5.00	0.16	2.80	0.31	0.20-0.40	0.10	2.82	0.063	6.35	0.35
	2nd half	9.00	0.20	5.36	0.35		0.15	4.16	0.063	7.60	0.62

although as a rule, when a limited income is available, the purchase of food is not one of the first things economized on. Our examiners, from questions and observations, reported that a deficiency in quantity probably existed in 15% of the whites and in 28% of the colored cases, corresponding in general with the figures previously given for poverty. It is to be remembered, however, that in a very appreciable number of cases, the onset of the first gastric symptoms of pellagra is followed, not preceded, by a change in diet. That is, with the accompanying anorrexia and the so-called billious attacks, as the pellagrin terms them, the diet is voluntarily diminished in quantity and changed in quality, the meats and fats being avoided most frequently.

*Change in Diet.*—Of 576 white pellagrins from whom information was obtained, 90% positively denied any deterioration either in the quantity or the quality of the diet in the years immediately preceding the first attack and only 18% of the colored cases had changed their diet in a manner that would indicate a lowering of its value.

*Fresh Vegetables and Fruits.*—Of the whites, 70% gave definite information to the effect that fresh uncooked fruits and vegetables in season formed part of the dietary, the determination for the colored race being practically the same, 60%. Considering that we are dealing with an urban industrial population, we feel quite certain that it compares favorably in this respect with any northern community.

*Proportion of Protein, Carbohydrate, and Fats.*—In order to obtain an accurate idea of the balancing of the diet, we have not been con-

tented with a mere statement from the examiners as to the quality of the dietary constituents, but have obtained statements as to the variety of the foods consumed, their quantity, and an average daily menu. In this way we have been able to approximate in a fairly satisfactory way the food value and the quality.

Protein: 1. High protein diet. As a high protein diet, we have considered the daily consumption of a pint of milk, 1 or 2 eggs, a daily ration of lean meat, bread made from wheat flour, and an adequate proportion of protein-rich vegetables.

2. Medium protein diet. As a medium protein diet, we have considered a glass of milk per day, lean meat 3-4 times per week, one-half dozen eggs per week, daily legumes, and either wheat or corn bread.

3. Low protein diet. In this, we have classed the well-known corn bread, fat meat, molasses, and vegetable diet, with practical exclusion of lean meat, milk, or eggs.

The first 2 diets, particularly when a sufficient amount of carbohydrates be added to the 2nd type, would under ordinary instances be considered a sufficient ration to maintain good health. The so-called low protein diet, we consider inadequate to maintain a proper state of nutrition.

The terms high and low carbohydrates and fats offer no difficulties in interpretation.

The relative percentages of the foods used, arranged for the periods of childhood, adult, and old age are graphically shown in Chart 9. It will be observed that a certain definite percentage of all the cases live on what may be termed a high protein diet, the maximum number on this diet being adults, an increase that holds true not only for the males but also for the females. Among the colored patients only few live on this type of diet.

Those living on the medium protein diet are greater in number, being about equal to those living on the low-protein ration. The females of both races living on these diets decrease in number for the several age periods. Relatively more colored females subsist on the inadequate or low-protein diet than white females.

The great majority of all patients lived on a high carbohydrate diet, no striking difference being observed either for the 2 races nor for the different age periods.

The number living on a high fat diet was relatively high.

*Variety of Protein Foods.*—When we now turn to the type of protein foods, the results shown in Chart 10 are obtained, in which the percentage of individuals in the different age periods partaking regularly of the protein foods tabulated, is indicated.

Here again, it will be observed for the whites that in adult life, both for the males and females, a great number partake of the various protein foods. For the colored race, the percentages indicate in general less consumption of proteins, with the exception of meat.

*Average Weight and Height of Pellagrous Children.*—Probably one of the most satisfactory methods of studying the effect of diet is the observation of the growth curve of the growing individual. In order to determine this relation, we have obtained the exact height and weight of all the school children of the city and have used their normal curves for purposes of comparison with the growth curves of pellagrous children (Charts 11-15).

The weights given include clothing. The proper deduction for shoes have been made from the height determinations. In practically all cases it will be observed that the height and weight for pellagrous children are somewhat below the normal from the 8th year on. The white children are one-half inch less in stature, 3.3 lb. less in weight for the boys, and 3.8 lb. less in weight for the girls. The colored pellagrous children are 1.1 in. less in stature, the colored boys 2.8 lb. less in weight, and the girls 7.2 lb. less than the average.

With the exception of the considerable difference in the weight of the colored girls (Chart 14), the deviations from the normal are approximately similar in extent to those observed for the schools in the poorer districts of the city, as compared to the normal for the entire city. In other words, a nutritional deficiency sufficient to cause a difference of 4 lb. in weight and one-half to 1 in. in height is found among the poor nonpellagrous as well as for the pellagrous.

Inasmuch as only 25 colored girls form the basis for the curve presented (only one-half as many as colored boys) it is possible that a larger number would have raised the average to a level more nearly equal to the value obtained for the other children.

It will be observed that at the first age recorded (7 years), the pellagrous children are equal in height and weight to the normal, while the more marked divergences occur after the 11th year, in all the charts.

## CONTACT

In our previous report covering 501 cases, 84.5% of the white cases and 57.9% of the colored cases were found to have originated after a constant contact with a previous case of pellagra.

For the purposes of the present study, we have grouped the contacts under headings similar to those used last year. The term previous case in family includes not only the cases in the immediate household, but also cases in immediate relatives with whom a constant contact is maintained; under 'adjacent houses' is understood not only the 2 adjoining houses, but the house immediately at the rear and across the street as well. The other terms are self explanatory. A certain number of cases have originated outside the survey area, and unless the patient knew of some contact, we were unable to trace the case properly; all these cases are included under the heading 'undetermined.' Were we to limit the contact determination to cases which originated in Nashville within recent years, we would be able to show a definite contact in more than 90% of the cases. Even in this large number of undetermined cases, we were able to obtain a definite contact history in 85% of the white cases and 80% of the colored cases. The proportion of the various possible contacts are shown in Chart 15. Similar to the observations of the first survey we noted many striking examples of the relation of one case to the development of others, but do not consider it essential for the points at issue to amplify the detail charts previously published.

## DISCUSSION

As a result of the surveys carried on during the past 2 years in the city of Nashville, we feel justified in drawing certain conclusions from fairly well established facts.

Pellagra has occurred in Nashville for 20 years or more. It is evident, however, that the earlier cases were few in number; since 1908, the disease has progressed rapidly, reaching a climax during the years 1914-1915. During the present season, the number of new cases has been smaller and the mortality less, despite the fact that with an augmented range of information, the recognition of deaths reported as pellagrous has been made more certain. We have then to explain first the increase and the decrease in the pellagra mortality.

Dietary diseases do not show such a curve unless there is a corresponding improvement in diet. We have no reason to believe that the great mass of people have consciously altered their diet in any way



during the past year. So profound an economic change as that involved in the purposeful changing of the quality of food cannot be accomplished in so short a period of time no matter how thorough the publicity used to obtain it. It might conceivably be altered more rapidly through a change in the economic condition. That, as a matter of fact, has taken place in so far as the agricultural South is concerned, in that crops have been abundant and excellent prices obtained for cotton, tobacco, and other staples. The industrial class, on the other hand, the class with which we are dealing, have had no such era of prosperity. The labor market has but been extended, except in so far as the North has absorbed a certain amount of surplus labor, wages have either not advanced at all, or only in a limited measure during the fall of 1916. The cost of food products, however, has increased at a most disproportionate rate.

If, on the one hand, the economic situation does not account for the decrease in pellagra during the present season, it fails, on the other, to account for the increase during 1914-1915. Food values remained remarkably constant during the entire period under consideration (1910-1915), and while unemployment was increased to some extent in 1914, the larger industries did not alter their forces, nor did the applications for charity increase to our knowledge.

When we consider the relation of the diet to pellagra, 2 factors stand out which are contradictory. On the one hand, a definite number of cases develop in individuals partaking of a diet as varied and as wholesome as could be demanded by any advocate of the dietary theory, and cases have come to our attention of pellagra developing in breast-fed infants of nonpellagrous mothers. On the other hand, at least half of the cases develop in persons living on a ration low in protein, high in carbohydrates, and monotonous in character. Admittedly, too, the pellagrous condition is favorably influenced by a change in diet.

It is unfortunate that the crucial experiment of Goldberger<sup>2</sup> and his associates producing pellagra in convicts on a restricted diet was made in a pellagrous community, so that the results are open to serious criticism. It would seem imperative that the most impressive part of the evidence be repeated under conditions free from any possible criticism.

<sup>2</sup> Goldberger and Wheeler: *Pub. Health Rep.*, Nov. 12, 1915.

Our observations of the growth of pellagrous children would not lead us to suspect any marked metabolic derangement as a causative factor. The younger children examined (7 years) are equal to the normal in height and weight, whereas the older children (8-15 years), many of whom have had pellagrous symptoms for years are under weight and below height, but to an extent apparently not exceeding that which obtains for the children of the schools in the poorer districts. It would seem that in these cases the disease process caused retardation, rather than that the disease resulted from a factor associated with the retardation.

As far as the epidemiology of pellagra is concerned, as studied under the conditions existing in Nashville, we cannot ignore the fact that the disease presents all the evidences of being in some way conveyed from one patient to another. It is practically a disease of the unsewered city areas, a family disease or almost as frequently a disease 'of the house next door,' and not only a family disease in the sense that the members live in the same house and eat the same food, but most frequently we have found that relatives, not living under the same conditions, but frequently associating, have one after the other succumbed to the disease. The fact, too, that cases develop in houses adjoining pellagrins, previously emphasized in the report of the Thompson-McFadden Commission,<sup>3</sup> is of great importance, because the chances for the wider contact, 'same block,' to prevail are naturally much greater than for the lesser number dwelling in adjacent houses.

The mode of occurrence of pellagra among the segregated negro colonies of the city is also of interest in this connection. To all intents and purposes the various groups live under identical economic conditions, and any variations in diet would be negligible. Pellagra occurs frequently in all except 1 of the groups (marked X on the map of the colored districts). The only explanation possible is that there is little pellagrous contact for this group. The other negroes live surrounded on all sides by pellagrous whites; this particular group is, however, cut off from the adjacent white pellagrous population by a wide railroad trackage and on the other side is adjacent to a well sewered nonpellagrous white zone.

<sup>3</sup> Siler, Garrison and MacNeal: Jour. Am. Med. Assn., 1914, 63, p. 1090.

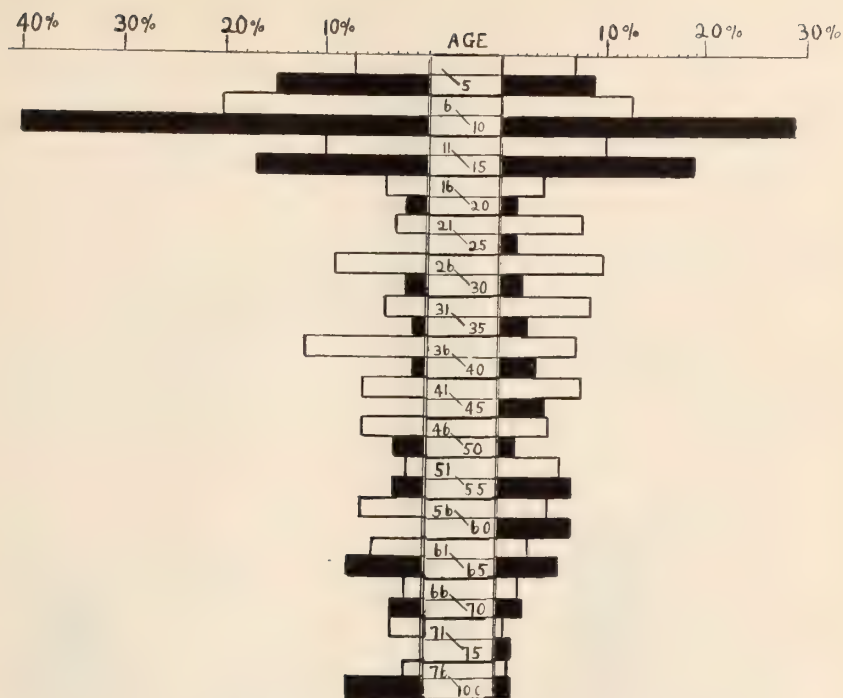


Chart 1.—Age incidence: Black columns = males; white = females.

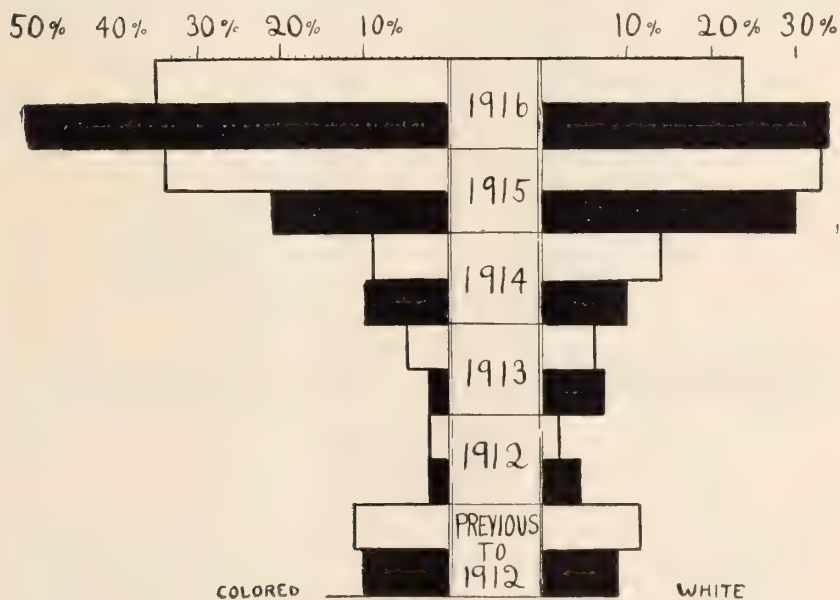


Chart 2.—Year of onset: Black rectangles = males; white rectangles = females.

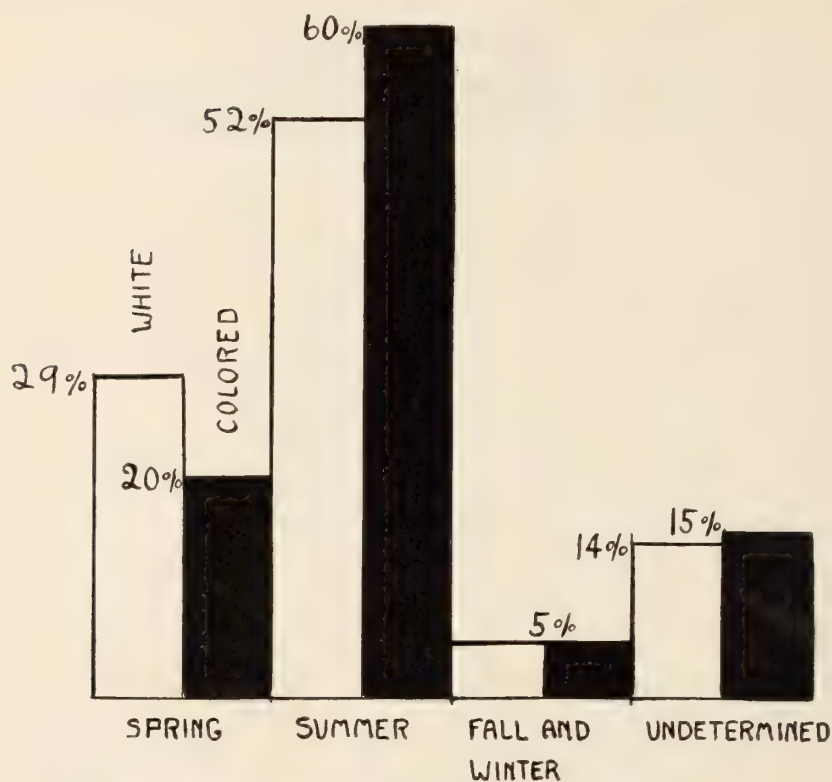


Chart 3.—Season of onset: White column = white race; black column = colored race.

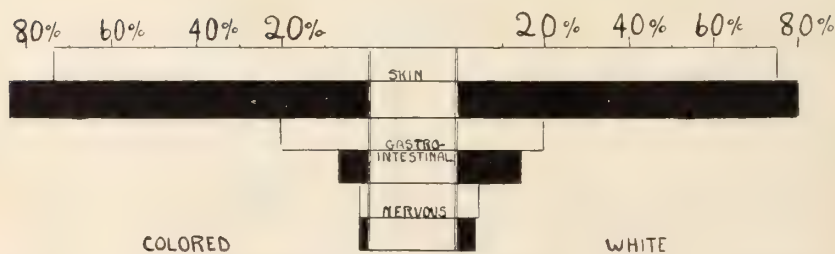


Chart 4.—Character of predominant lesions: White columns = females; black columns = males.

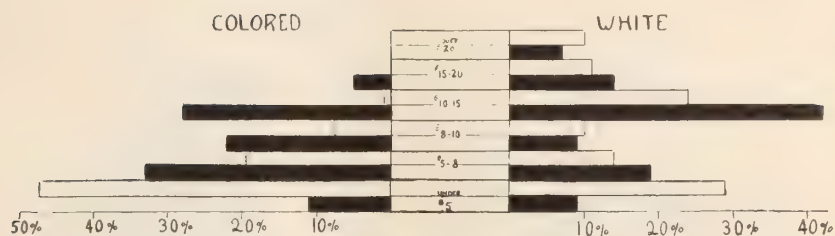


Chart 5.—Average wage per week of pellagrins: White columns = females; black columns = males.

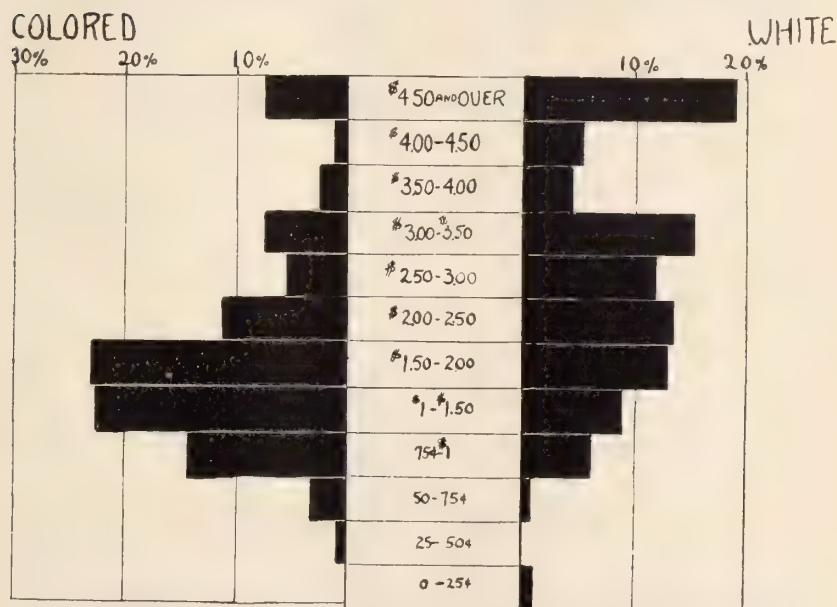


Chart 6. Average income per week of pellagrins.



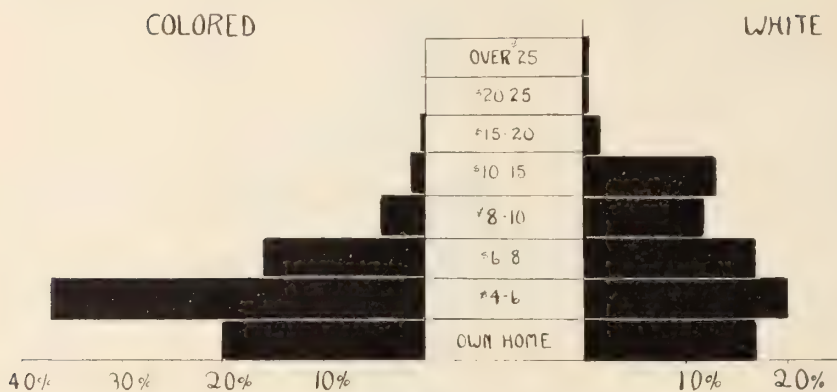


Chart 7.—Average rentals of pellagrins.

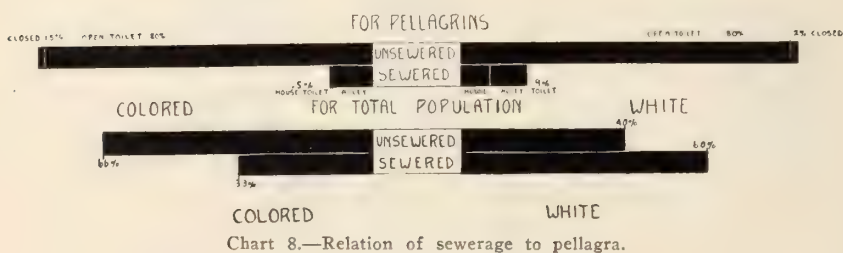


Chart 8.—Relation of sewerage to pellagra.

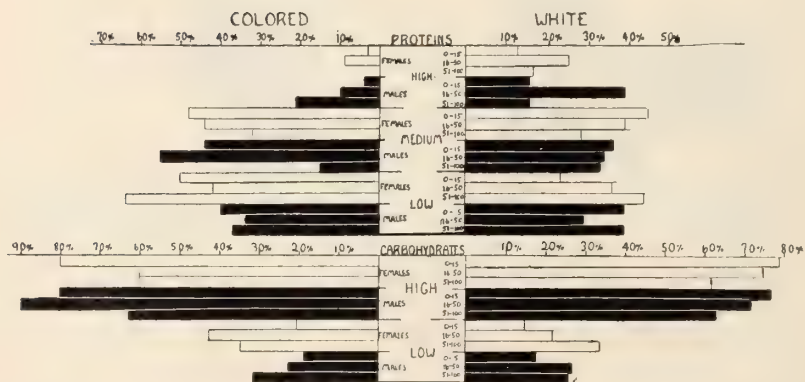


Chart 9.—Relative proportion of proteins, carbohydrates, and fats in the diet of pellagrins: White column = females; black columns = males.

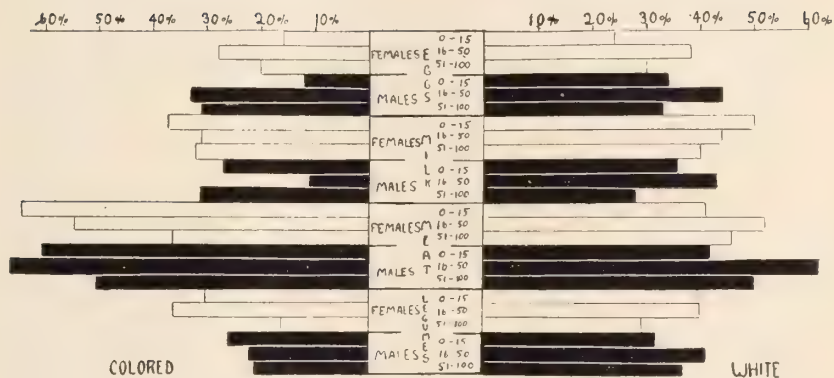


Chart 10.—Type of protein in the diet of pellagrins: White columns = females; black columns = males.

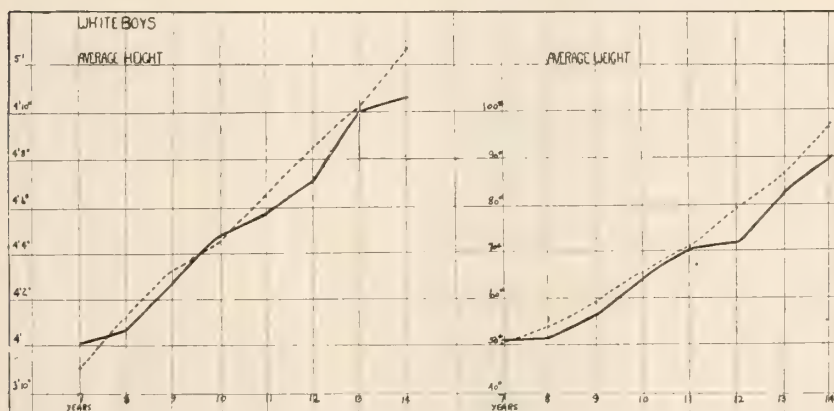


Chart 11.—Growth curves of normal and pellagrous white boys: Dotted line = normal (average for all schools); solid line = pellagrous children.

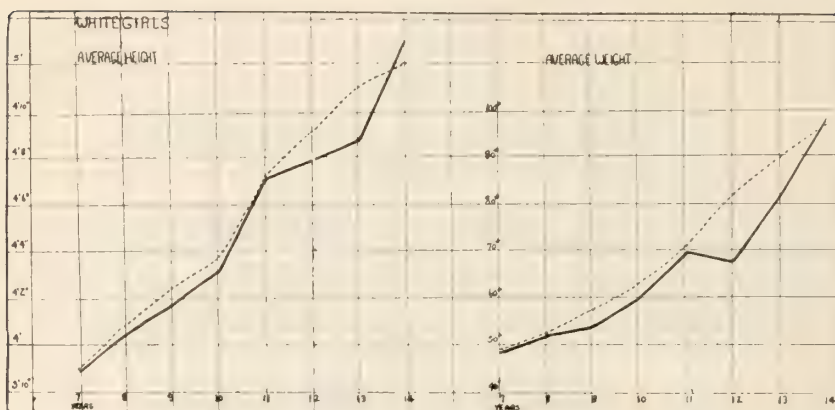


Chart 12.—Growth curve of normal and pellagrous white girls: Dotted line = normal (average for all schools); solid line = pellagrous girls.

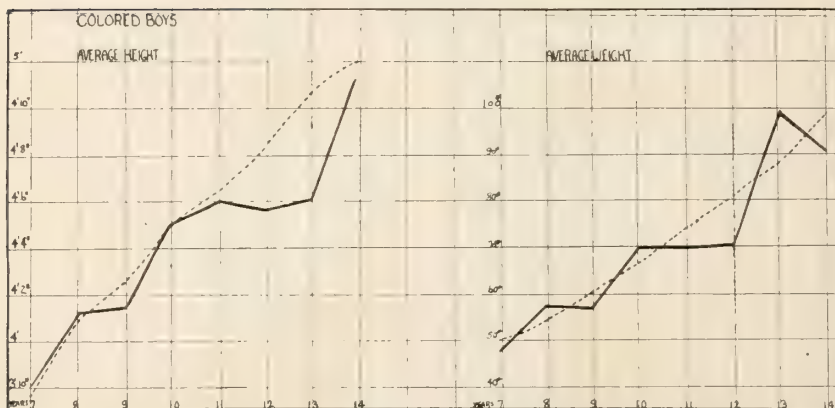


Chart 13.—Growth curve for normal and pellagrous colored boys: Dotted line = normal (average for all schools); solid line = pellagrous boys.

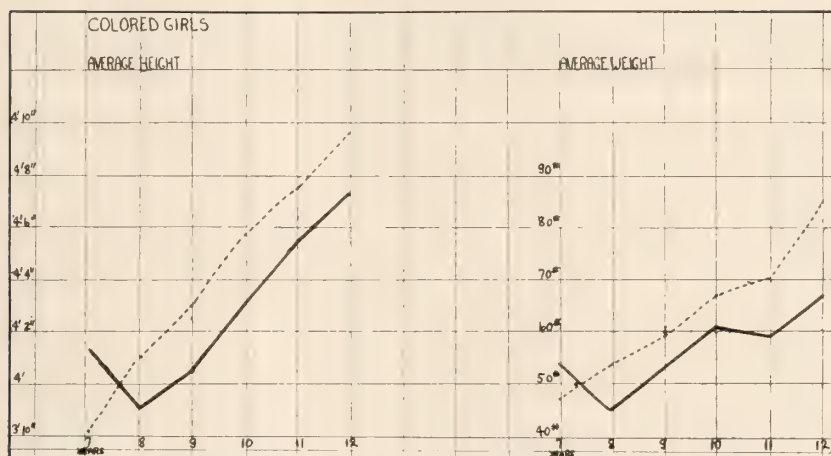


Chart 14.—Growth curves for normal and pellagrous colored girls: Dotted line = normal (average for all schools); solid line = pellagrous girls.

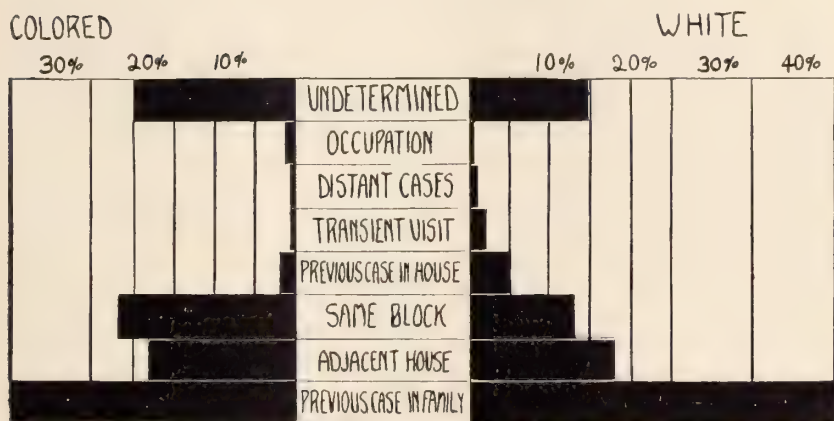


Chart 15.—Proportion of varieties of contact to the development of pellagra.

# DEVELOPMENT OF ANTIBODIES FOR BACILLUS TYPHI-EXANTHEMATICI IN TYPHUS FEVER CONTACTS \*

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In a previous publication,<sup>1</sup> 2 observations were reported which indicated that individuals who are exposed to typhus fever may react with the production of specific antibodies without having had any clinical evidences of the disease. During the past year, the number of such observations have multiplied, so that it now seems as if the phenomenon were by no means uncommon. These observations are unquestionably of considerable etiologic as well as epidemiologic significance; it has, therefore, been thought advisable to report them now in detail.

The first observations were made in New York, and for the serologic work in these cases I am indebted to Dr. Peter K. Olitsky; the later ones were made while studying a recent typhus epidemic in Volhynia, Russia. Complement fixation tests were made with a typhus antigen, consisting of the clear Berkefeld filtrate of a 24-hour autolysate of *Bacillus typhi-exanthematici*, previously killed by heating to 60 C. for an hour (for detailed technic, see paper by Olitsky<sup>1</sup>). This antigen is absolutely specific, giving positive reactions only with the serum of typhus convalescents and in the group of cases reported in this paper. Uniformly negative results have been obtained in 150 control cases, including a great variety of acute and chronic febrile diseases and other pathologic conditions.

The agglutination tests were carried out with a polyvalent agglutinin whenever possible, and the microscopic method was used for reasons, detailed in a previous paper.<sup>1</sup> Agglutinins, as a rule, first appear in the blood during the second week of typhus fever, and increase rapidly at the crisis. On the average, the maximum titer is attained during the second week of convalescence; the agglutinins then

\* Received for publication February 2, 1917. Work done under the tenure of a George Blumenthal, Jr., fellowship in pathology. Most of the observations reported in this paper were made while carrying on investigations upon typhus fever in Eastern Europe during 1915-1916. The expedition of Dr. Plotz and the author was financed by the directors of the Mount Sinai Hospital and carried on its work under the auspices of the American Red Cross.

<sup>1</sup> Plotz, Olitsky, and Baehr, Jour. Infect. Dis., 1915, 17, p. 1.



gradually diminish, but disappear from the blood, as a rule, only after many months. As I was recently able to show,<sup>2</sup> the curve of the specific agglutinins in typhus fever is characteristically an immunity curve, rising with recovery and the development of immunity, persisting for a long time, then gradually and slowly diminishing.

On nontyphus cases, Dr. Olitsky, Dr. Plotz, and I have now done over 200 agglutination tests. Occasionally, nonspecific agglutination has been observed in low dilutions of serum, a phenomenon common to most bacteria. Only twice have we seen an agglutination with serum of a nontyphus case in a dilution as high as 1:50. In higher dilutions, agglutination has only been observed in individuals who have or have had typhus fever, or who have been vaccinated with *Bacillus typhi-exanthematici*, and in the group of cases which forms the subject of this report. This last group includes all those patients in whom we have found a high antibody content and who clinically have not had typhus fever.

In view of the otherwise negative serologic findings in nontyphus cases, these observations are interesting and important. The first two cases were reported in a previous paper.<sup>1</sup>

CASE 1.—Toward the end of 1914, after carrying on experimental and other work on typhus fever for over six months, Dr. B. experienced some slight malaise and general muscular pains for a few days, but they were not marked enough to require any attention. Three or 4 weeks later, his blood was examined serologically, preliminary to the proposed administration of vaccine. Much to our surprise, the serum gave a ++++ complement-fixation with typhus antigen and agglutinated typhus bacilli in a dilution of 1:500. As we had observed serologic reactions of this degree only in individuals who were convalescing from typhus fever, we had reason to suspect that Dr. B. had recently passed through an infection, although he had shown only slight clinical manifestations of illness. This was in a manner confirmed by the fact that, as in convalescents from typhus fever, the serologic phenomena gradually diminished in intensity after the first month, so that by the end of 4 months, both complement-fixation and agglutination became practically negative.

TABLE 1  
RESULTS OF SEROLOGIC TESTS

Time of Test	Complement-Fixation	Agglutination
Dec. 15, 1914	++++	1:500 +
Dec. 21, 1914	++++	1:200 +
Mar. 1, 1915	++	1:50 +
Apr. 17, 1915	negative	1:20 +

Previous to these observations, Dr. B. had been in contact with patients suffering from the mild New York typhus, but usually only after their clothing had been removed and they had been bathed. Dr. B. also bled typhus guinea-

<sup>2</sup> Jour. Infect. Dis., 1917, 21, p. 21.

pigs and monkeys almost daily for the purposes of study, virus transmission, etc. No special precautions were taken to avoid infection, the virulent blood of infected animals often remaining on his hands throughout a series of experiment lasting over an hour. That an infection can be produced in human beings by direct inoculation of typhus blood has been amply demonstrated by Moczutkowski,<sup>3</sup> Otero,<sup>4</sup> Yersin and Vassal<sup>5</sup> and others. So it is possible that Dr. B.'s infection could have occurred without the usual intermediary agency of the body louse.

CASE 2.—With this observation in mind, similar examinations were made on two nurses, Miss L. and Miss H., who had just returned from Serbia where they had been nursing typhus fever patients during the big epidemic of 1914-1915. Many of the patients under their care had been covered with vermin. Miss L. believed that she had been repeatedly bitten by lice and that on at least two occasions she had removed lice from her clothing. The serologic studies on Miss H. were negative. The serum of Miss L. gave a +++ fixation with typhus antigen and firmly agglutinated typhus bacilli in a dilution of 1:200.

CASES 3 AND 4.—A Macedonian immigrant left the town of Florina, near Monastir, in Macedonia, on March 1, 1915, arriving in Salonica on the same day. On March 2, he left Salonica on a packet boat for Piraeus, and on March 3, sailed from Piraeus on a Greek steamer for New York. The steamer touched at Kalamata, Patras and Algiers, enroute, and arrived in New York, March 23. He immediately left New York by rail for East Syracuse, arriving there March 24. He experienced the first symptoms of illness, April 7 and was acutely ill, April 9, the disease ending with the defervescence of temperature, April 19, the twelfth day of illness. The case was definitely diagnosed as typhus fever by Dr. John L. Kantor<sup>6</sup> who had had experience with the disease in New York. On the tenth day of illness, complement-fixation was negative, but his serum agglutinated typhus bacilli in a dilution of 1:100. Five days after the crisis his complement-fixation was still negative, but his serum agglutinated in a dilution of 1:1800. No further examinations were made.

The mode of infection in this case was definitely ascertainable. During the two weeks following February 21, there were six deaths from typhus fever in Salonica. According to the patient's story he was free of vermin until he boarded the packet boat at Salonica. Both the packet boat and the steamer on which he subsequently traveled to New York were in a filthy condition, and by the time the vessel arrived in New York, most of the steerage passengers harbored body lice. As his illness began on April 7, the infection must have occurred on shipboard.

Four other Macedonians roomed with him in Syracuse, one of whom had been with him on his sea journey. None of them subsequently developed the disease. The blood of all 4 was examined serologically on April 27. In 2 of them, complement-fixation and agglutination were negative. The other 2, however, had a ++ complement-fixation and their serum agglutinated the typhus bacilli in dilutions of 1:200 and 1:300, respectively.

<sup>3</sup> St. Petersburg. med. Wchnschr., 1900, 25, p. 30. Allg. med. Centr.-Ztg., 1900, 69, p. 1055.

<sup>4</sup> Memoria presentata a la Acad. de Medicina de Mex., Mexico City, 1907.

<sup>5</sup> Philippine Jour. Sc., 1908, 3, p. 131.

<sup>6</sup> Jour. Infect. Dis., 1915, 17, p. 522.

CASE 5.—W. P. had charge of the animals used by us at the Mount Sinai Hospital in New York and also assisted us with all our experimental work. The mode of infection in his case is as obscure as was that of Dr. B., and might also have occurred without the agency of the louse. It is of interest to note that he handled dozens of typhus-infected monkeys and guinea-pigs daily, and also frequently came in contact with virulent typhus blood.

TABLE 2  
RESULTS OF SEROLOGIC TESTS

Time of Test	Agglutination	Complement-Fixation
April 27, 1915.....	1:500	++++
Sept. 10, 1915.....	1:200	++++

CASE 6.—Dr. U. has practiced medicine in Mexico for many years. During the past year, there has been a large epidemic of typhus fever in Mexico City and Dr. U. treated many cases. About six months ago, his wife and his daughter contracted the disease, but Dr. U. remained absolutely well. Recently while on a visit to New York, we had an opportunity to examine Dr. U.'s blood. It showed a complement-fixation of +++ and an agglutination of 1:200.

The conditions in Russia were especially favorable for the investigation of this problem because of the careful precautions taken by the Austro-Hungarian military authorities for the quarantining of typhus fever patients and those who had been in contact with them. Volhynia and Russian Poland were studded with typhus hospitals and hospitals for epidemic diseases. On the outbreak of typhus fever in a community, the individuals who had been in contact with the patients were immediately confined in the quarantine building of the typhus hospital. Here they were in the charge of a nurse; they were visited twice daily by a physician, and their temperatures were recorded 3 times a day. On the appearance of the first prodromal symptoms of illness or the slightest rise in temperature above the normal, the suspect was immediately transferred to the observation ward of the hospital.

CASE 7.—On January 9, a family of six people, father, mother and 4 daughters, were sent to the typhus hospital from a village where typhus fever had recently become epidemic. On admission, 3 daughters were in the second week of a typical typhus fever of moderate severity and the mother was in the third day of the illness. The mother's illness lasted 15 days, but was for the most part so mild that if the patient had not been confined to the hospital, hers would undoubtedly have been an ambulatory case. All 4 patients developed agglutinins in their blood toward the end of their illness.

On the ninth day of the illness, the mother's serum agglutinated the typhus bacillus in a dilution of 1:200; on the fourteenth day, 1:200. During convalescence, agglutinins were found in the blood in a dilution of 1:500, on the third day; 1:300, on the seventh day, and 1:300, on the eleventh day. Agglutinins were found in the blood of the first daughter on the sixteenth day of the illness in a dilution of 1:100; on the third day of convalescence, 1:100; on the seventh day of convalescence, 1:50. The blood of the second daughter was not examined during illness; on the second day of convalescence, the

serum agglutinated the typhus bacillus in a dilution of 1:500; on the seventh day, 1:200. In the blood of the third daughter, agglutinins were found on the fourteenth day of the illness in a dilution of 1:50; during convalescence, it was not examined.

On admission to the hospital, only the father and the youngest daughter were free from the disease. The girl remained well and her temperature was normal until the tenth day after her admission. On that day she complained of headache and chilly sensation and the temperature rose to 37.8 C. On the following day, she had a severe chill and the temperature rose to 40.2 C. The rash first appeared on the evening of the fourth day and from then on her disease ran a typical course of moderate severity, ending by crisis on the fifteenth day. The typical clinical picture was confirmed by the bacteriologic and serologic findings. Three blood cultures, taken on the first, fifth, and eleventh days of the disease, were all positive; in the first culture, taken at the time of the initial chill, 168 colonies developed in 12 c.c. of blood. Agglutination of *Bacillus typhi-exanthematici* by the patient's serum was negative before the onset of the illness; positive 1:50, on the eighth and twelfth days, and positive 1:100, on the first and fifth days of convalescence.

The only member of the family who failed to develop the disease was the father. He remained well and the temperature never rose above normal during the entire 4 weeks after exposure, during which he was in quarantine. At this time, the blood was examined 3 times for agglutinins. On the second day of admission, the results were negative; on the seventh day, agglutinins were found in a dilution of 1:50; on the twentieth day, 1:100. So, of the 2 members of the family who were still well at the time of admission, one subsequently developed the disease, whereas the other, who remained well, simultaneously developed in his blood specific agglutinins for *Bacillus typhi-exanthematici*.

CASE 8.—Two Austro-Hungarian nurses belonging to the staff of a mobile epidemic hospital situated on the Russian-Galician frontier were assigned to temporary duty in a distant Russian village where typhus fever was epidemic. They lived in a small hut with a peasant family, all the members of which were severely ill with typhus fever. One did the nursing during the day, the other was on duty at night. The patients were infested with lice, and the nurses were unable to protect themselves from the vermin. Under these primitive conditions they lived for three weeks, when they were relieved and re-assigned to hospital duty. Six days later one of the nurses developed typhus fever. We saw her on the eighth day of her illness, at which time she had an extensive and typical rash and presented the characteristic picture of the disease. A blood culture taken on this day was positive, 3 colonies of *Bacillus typhi-exanthematici* developing in 10 c.c. of blood. Her serum also agglutinated the typhus bacillus in a dilution of 1:100.

The second nurse had similarly been exposed to typhus fever for 3 weeks, but had subsequently remained well, nor had she previously ever been ill. Her blood was examined on the same day as that of the sick nurse, two weeks after being relieved from her previous typhus duty. It agglutinated the *Bacillus typhi-exanthematici* up to a dilution of 1:500.

CASES 9 TO 12.—A family of eleven individuals lived together in a typical, squalid peasant hut, situated in the Russian village of Rahozno, where typhus fever had recently become epidemic. In the early part of December, 1915, the mother and 4 of the children became severely ill with typhus fever. The entire family was then immediately transferred to the typhus hospital, the sick



individuals to the wards, and those who had remained well to the quarantine building. The 5 sick members of the family presented the typical picture of the disease, the mother dying December 19. The autopsy revealed the usual more or less negative findings of typhus fever. The remaining six members of the family were under constant medical observation both during the 4 weeks' sojourn at the hospital, and subsequently at their home, and at no time did they have the slightest rise in temperature. Four weeks after the death of the mother, we were able to procure some blood from 5 of the 6 members; their serum agglutinated *Bacillus typhi-exanthematici* in the following dilutions:

Wasył .....	1: 50 +
Alexandra .....	1: 200 +
Hanna .....	Negative
Wartha .....	1: 100 +
Maria .....	1: 200 +

CASE 13.—The Jasinoſky family, neighbors of the Choročovskys, consisted of seven individuals, and they had lived in one room since the end of September, 1915. Five of the 7 were subsequently ill with typhus fever, 4, during the month of October and the fifth, during November. The existence of typhus fever in this house was not discovered by the military authorities until the beginning of November, when the fifth member of the family became ill. The entire family was then brought to the hospital, "entlausst," and kept under observation. Here, the man who was still ill, ran the typical course of typhus fever, ending in recovery on November 15th. Two months later, we had the opportunity of examining the blood of 5 members of this family. Of these, 2 had had the disease in October and one in November. Agglutination of *Bacillus typhi-exanthematici* was obtained with their serum in the following dilutions:

Maxim .....	1: 100 +
Wasył .....	1: 150 +
Andreas .....	1: 50 +

The results of similar examinations made upon the 2 members of the family who had escaped the illness were as follows:

Priska .....	1: 300 +
Stefan .....	Negative

CASES 14 AND 15.—The head of another family, T., had typhus fever from November 10 to 25. In the second week of his illness, he and the members of his household, his wife, sister-in-law and mother-in-law, were transported to the typhus hospital, where they were under observation for 4 weeks. Previous to that time, the family had lived in a 2-room house, T. and his wife in 1 room and the sister-in-law and mother-in-law in the other. The former room also served as a living- and dining-room for the family. During their stay at the hospital, none of the family, with the exception of T., showed the slightest rise of temperature, nor did they suffer from any illness either before or after this time. Two months after T.'s illness, blood was secured from these 3 members of the family and examined for agglutinins, with the following results:

Wife .....	1: 50 +
Mother-in-law .....	Negative
Sister-in-law .....	1: 100 +

CASE 16.—At the typhus hospital in Volhynia, in which most of these observations were made, only people who had once had typhus fever were employed as doctors, nurses and orderlies. But in addition to this staff, there were 11



nonimmunes, men who had not had the disease, employed about the hospital as mechanics, carpenters and general workmen. These men had their living quarters apart from the rest of the staff, but it was not always possible to keep them from coming in contact with men on duty in the wards or in the reception room of the hospital, or with those whose duty it was to transport typhus patients in ox-carts from the village to the hospital. In view of their residence in a typhus hospital, the possibility of infection could not, therefore, be avoided, and for this reason the blood of these 11 men was examined for agglutinins. All were negative with one exception; a carpenter who had been working at the hospital for 4 months, but had been well during that entire time. His serum strongly agglutinated *Bacillus typhi-exanthematici* to a dilution of 1:200.

CASE 17.—Another man, M., denied having been ill. On admission to the quarantine hospital, his temperature was 38 C., but was normal thereafter. Upon examination, his skin showed what the clinicians at the hospital thought was suspiciously like a recently faded typhus rash. The patient also looked ill and worn, as if he had recently been through an illness. Upon questioning his wife, she also denied that he had been ill, but said that for the last 10 days he had acted stupidly, though he had otherwise gone about as usual. From the general appearance of the patient, the suspicion was very strong that he had just been through a mild ambulatory typhus, and the authorities, therefore, sent him to the typhus hospital. Their suspicion was then still further confirmed by the agglutination tests which were made without any knowledge of the foregoing facts. Tests made on 2 occasions showed a strong agglutination in a dilution of 1:100. It was later learned, through subsequent investigation, that his sister-in-law's husband had had typhus fever about 4 weeks previous. But when an attempt was made by the military authorities to quarantine the family, this sister-in-law had escaped and had gone to live with the M.'s family.

CASES 18 to 20.—The attendants working in the reception room of a hospital where typhus fever patients are daily being admitted are especially liable to infection. Their duty is to "cleanse" the patient, remove his vermin infected clothing and bathe and shave him. As a rule men who have had typhus fever are assigned to this duty. But often, especially at the onset of an epidemic, the demand for such people has been greater than the supply, and nonimmunes were then employed. Many of these men succumbed to infection. But we have found 3 such individuals who, in spite of the constant exposure to infection, have never had any symptoms of illness, and in their blood we have found agglutinins for the *Bacillus typhi-exanthematici*. In 2, agglutinins were found in a dilution of 1:100; in the third, in a dilution of 1:200.

#### SUMMARY

These 20 observations include all the cases observed by us, which did not have typhus fever clinically, but in whose blood specific antibodies in high titer were found. Every one of these 20 individuals was found to have recently been in intimate contact with typhus fever. They were either doctors, nurses and hospital attendants who were handling typhus fever patients, or they were friends or members of families in which typhus fever had recently occurred. The latter had

lived in the same room with their sick relatives or friends, or had been in close association with them. The significance of these observations can be really appreciated when we realize that in over 250 other non-typhus controls, who, with few exceptions, had not been in contact with typhus fever patients, complement-fixation was never observed and specific agglutinins were not demonstrable in a dilution above 1:50.

Of these 20 typhus contacts, 3, after their exposure to infection, had had vague, general symptoms indistinguishable from an influenza. All the others had been well. Shortly after their exposure, the sera of these contacts were found to contain specific antibodies in amounts otherwise only observed by us in individuals who have recently recovered from typhus fever. These findings permit of only 1 interpretation, that at the time of their exposure, the contacts had actually been infected with the bacilli, but in quantities insufficient to induce the clinical manifestations of the disease. The phenomenon is comparable to what one would expect from the inoculation of a subinfective dose of the virus or of virulent bacilli, especially if such an inoculation were frequently repeated.

Whether such contacts after they have developed a high antibody titer in their blood are permanently immune to the disease, cannot, of course, be ascertained with any positiveness. We are only in a position to say that most of these people continued to live in the midst of a typhus epidemic for some months and none of them developed the disease. In this regard, however, some of the experimental work which has been done on monkeys is undoubtedly of some significance. Nicolle, Conor and Conseil,<sup>7</sup> Ricketts and Wilder,<sup>8</sup> and Anderson and Goldberger<sup>9</sup> have independently noted that monkeys which have been exposed to the bites of infected typhus lice may subsequently develop an immunity without having had fever or any other sign of illness. It is therefore quite reasonable to suppose that the same thing may occur in human beings after a similar exposure to infection, especially in view of the additional serologic evidence which we have accumulated.

We have now met with 20 such instances during the course of our work, without having made any systematic search. Such individuals who have become immune without actually having had the disease cannot therefore be very uncommon. And they are a serious danger epidemiologically, for they may act as carriers of the infected lice and

<sup>7</sup> *Compt. rend. Acad. d. sc.*, 1909, 149, p. 149.

<sup>8</sup> *Jour. Am. Med. Assn.*, 1910, 54, p. 1304.

<sup>9</sup> *Collected Studies on Typhus Fever*, Bull. 86, Hyg. Lab., U. S. P. H. S., 1912.

be the agents of their distribution throughout a community. This is no theoretical consideration, for da Rocha Lima has recently demonstrated experimentally that typhus lice when kept alive by repeated feedings on typhus immune individuals do not lose their virulence for a long time.

The general immunologic principle deducible from the observations which are here reported are probably generally applicable to other infectious diseases besides typhus fever. That mild abortive forms of the various infectious diseases are followed by an immunity is generally recognized. The suspicion has also been voiced that people may become immune following a simple exposure to some of the infectious diseases, even though at the time they have shown no signs of illness. Up to the present, however, there has been no direct scientific evidence that this does occur.

The observations recorded in this paper are of importance from another aspect. The observation that individuals after exposure to typhus fever may fail to develop the disease, and yet may react with the production of specific antibodies against *Bacillus typhi-exanthematici* is incontrovertible evidence as to the etiologic significance of this organism. The phenomenon cannot possibly be explained on any other ground than that the *Bacillus typhi-exanthematici* is the cause of typhus fever.

# FECAL EXAMINATIONS OF A REGIMENT INFECTED WITH BACILLUS PARATYPHOSUS A, WITH SPECIAL REFERENCE TO NORMAL CARRIERS \*

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The Fourteenth Infantry, N. G. N. Y., returned last fall from the Mexican border badly infected with paratyphoid (A type). Dr. Charles W. Berry has reported the epidemiology of this outbreak.<sup>1</sup> The onset of paratyphoid fever was foreshadowed and accompanied by many cases of enteritis with diarrhea. As far as we know, however, *Bacillus paratyphosus A*, in contrast to other members of the paratyphoid-enteritidis group, does not cause acute enteritis; paratyphoid A infections, clinically and epidemiologically, are closely allied to typhoid fever. The close resemblance of this outbreak to water-borne typhoid epidemics, which are frequently accompanied by outbreaks of diarrhea, indicates strongly that it was water-borne.

In all, about 215 cases were diagnosed among 1000 men; the total incidence of the disease was probably higher. Because of this high incidence, a general fecal examination was undertaken after the return of the regiment to the New York armory, preliminary to discharge. Altogether, 843 men were examined; this paper presents an analysis of the results of these examinations. The specimens were collected under the supervision of Captain Dr. Charles W. Berry, who also supplied the histories. Special cases were investigated further by Dr. E. F. Marsh. The bacteriologic examinations were made by Ann Kuttner, Elsie Schumm, and Margaret Kelley; the final identification of the strain isolated was made by Lawrence A. Kohn.

The number of cases examined is too few to establish the incidence of excretion of bacilli at different periods after infection, but the results are suggestive. The percentage of positive findings during the first week is much higher than in typhoid fever. During the second week, it about equals the highest percentage (53%) which has been recorded

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<sup>1</sup> Med. Rec., New York, 1917, 91, p. 135.

for typhoid (Gaeghtens and Brüchner<sup>2</sup>). In the third and fourth weeks, the percentage falls, whereas all investigators have found an increased excretion after the first week of typhoid fever which reaches its height, as a rule, during the third week. This marked difference is probably correlated with differences in the intensity and extent of intestinal lesions in the two diseases and not accidental, due to the small number of cases examined. It has been possible to repeat the examinations on only a few of these men, so that I can give no data on the incidence of chronic convalescent carriers. As individual case histories are not available, the results cannot be analyzed in relation to the time of disappearance of the fever, and as the duration and intensity of the disease varied so considerably, an average febrile period cannot be used. Besides the cases tabulated, one man with positive fecal results developed a pyonephrosis 12 days later. He had not been ill previously and, unfortunately, it was not possible to determine whether this condition was due to a renal localization of *B. paratyphosus* A.

TABLE 1  
RESULT OF EXAMINATION; CASES OF PARATYPHOID FEVER

Time of Examination	Examined (No.)	Positive (Per Cent.)
Incubation (period of one week before onset) .....	13	15
At onset or during first week .....	6	83
During second week .....	2	50
During third week .....	9	33
During fourth week .....	10	20
During fifth week .....	12	15
During sixth and seventh week .....	5	20

Exclusive of the cases cited, there remains a total of 786 men. Of these, 32, or 4%, gave positive fecal results. Twenty-one were re-examined 10 to 12 days later and 9 were again positive. The histories of these men were carefully investigated, and no record of illness before or after the time of examination was found. Although the absence of any history of illness is not conclusive evidence that none of them had had mild transient symptoms, it is fairly conclusive for the following reasons: At the border the camp conditions were rigorous and the temperature high, and the added strain of drills and forced marches makes it unlikely that any man who was even slightly ill would not have reported to the medical officer. The men were from urban life and occupations, and were not hardened to the strain of

<sup>2</sup> Cited by Ledingham and Arkwright, *The Carrier Problem in Infectious Diseases*, 1912, p. 10.



actual field service. As soon as the presence of a febrile epidemic was recognized, the medical supervision became rigid, and a general thermometer inspection was instituted. After the return of the regiment it is unlikely that any illness would have been overlooked, as the men were closely observed until the time of discharge. Subsequent to discharge, which took place after all the fecal examinations were completed, no case of illness was reported in this group of men.

It therefore seems justifiable to put these men in the class of normal carriers, that is, in the group of intestinal carriers who without evidence of disease, harbor and excrete bacilli for a variable, but usually short, length of time. As such, the percentage (4%) is very high. Doubtless, it is directly proportional to the high degree of exposure to infection and to the relatively low virulence of the infecting type, which, in spite of intestinal multiplication, did not go on to general invasion. It is hardly necessary to emphasize the important rôle these men would have played in the spread of infection had they remained unrecognized, which would have been the case had general fecal examinations not been instituted.

The methods employed in the examinations were the use of brilliant-green agar previously reported,<sup>3</sup> the tentative agglutination from the plate by the macroscopic slide method, using a paratyphoid A serum, and the isolation of positive colonies with the Russell medium, followed by final cultural and agglutinative identification. None of the strains isolated fermented xylose, and all were agglutinated by the type serum. As a strain isolated from a member of this regiment was at our disposal at the beginning of the investigations, the brilliant-green medium was standardized directly against the infecting organism. From 0.35 to 0.5 c.c. of a 0.1% solution of brilliant green to 100 c.c. of agar gave good results, the amount applicable varying with different batches of agar. Because of the relatively marked resistance of the type and the large volume of work, only one strength of dye was used, and the high concentration of brilliant green made heavy inoculation possible. Two dye and 2 Endo plates were used for nearly all samples. The comparative results, where recorded, were: dye agar, 48 positive, and Endo plates, 25 positive, or 52% of total positive findings. Undoubtedly, more elaborate technic would have increased, to some extent, the total number of positive findings.

<sup>3</sup> Krumwiede, Pratt and McWilliams, *Jour. Infect. Dis.*, 1916, 18 p. 1.

## SUMMARY

Examination of the stools of 57 men with paratyphoid fever gave the highest percentage of positive results (83% ) during the first week of the disease.

In a series of 786 men exposed to infection, 4% of carriers of *B. paratyphosus* A had no history of a paratyphoid infection and may be classed as normal carriers. The duration of this condition was probably short in most instances.

The use of a selective plating medium for isolation resulted in a marked increase in positive results.

## ISOLATION OF TYPHOID BACILLI FROM URINE\*

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The detection of chronic typhoid carriers has come to be recognized as a factor of prime importance in any campaign to reduce the typhoid rate in a community. The agglutination test may cast a suspicion on certain persons, but the ultimate discovery of the carrier must rest on the isolation of typhoid bacilli from the feces and urine. The isolation of typhoid bacilli from stools has been extensively studied and many special methods have been devised; the urine, however, has received but scant attention, probably because the typhoid bacilli, when present at all, are usually in enormous numbers and can then be readily isolated by inoculating a drop of the urine on Conradi-Drigalski or Endo plates. It would seem desirable, however, to be able to recover typhoid bacilli also from the smaller percentage of urines that do not contain them in large numbers. This is especially true, since the urine sometimes contains typhoid bacilli when the feces are negative.

Hospitals are beginning to require several negative reports on the feces of typhoid patients before they are discharged; obviously, it is equally important that the urine should be shown to be free from typhoid bacilli. This, then, also shows the need of a simple, practical, and yet delicate method of recovering typhoid bacilli from urine. We have attempted to devise such a method.

Urine for routine examination will not be collected and delivered to the laboratory free from extraneous bacterial contaminations. For enrichment it is necessary, therefore, to provide a medium that will allow the typhoid bacilli to multiply, while inhibiting to a certain extent the growth of other bacteria. In many instances the urine itself was found to serve this purpose, but occasional specimens of urine inhibited the growth of the typhoid bacilli; it then was necessary to dilute the urine in order to be sure that it would always allow the multiplication of the typhoid bacilli. Parallel experiments were performed to determine whether the addition of dextrose or broth would yield better results than simply diluting with distilled water or saline solution.

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Our first experiments were carried out with nutrient broth of reaction + 1, and it was found that 1 part of the broth to approximately 2 parts of the typhoid urine gave the best results; if more broth was added, the other bacteria were likely to overgrow the typhoid bacilli.

The diluted urine is by no means a perfect enrichment medium for the typhoid bacillus; other bacteria multiply in it also. We therefore inoculate, after 24 hours' incubation, upon solid medium that is selective for typhoid bacilli. For this purpose, we employ the eosin brilliant-green agar of Teague and Clurman.

This medium is prepared as follows: 1 pound of chopped beef is soaked in 1000 c.c. of distilled water in the ice-box over night. The fluid is squeezed through cheese cloth, heated and passed through filter paper. To the filtrate are added agar (1.5%), Witte's peptone (1%), and chemically pure sodium chlorid (0.25%). This mixture is heated in the autoclave at 15 lbs. pressure for 20 minutes. The reaction is brought to +1 (hot titration) and the medium is cleared with egg white and filtered through cotton. It is then placed in flasks in amounts of 200 c.c., sterilized in the autoclave, and stored for use. To 50 c.c. of the agar are added  $\frac{1}{2}$  gm. lactose, 1 c.c. of 3% eosin solution, and 1 c.c. of 0.2% brilliant-green solution. The agar is shaken and poured into Petri dishes.

Whatever selective medium is being used for typhoid stools in a given laboratory should be employed for the urine also, since the full effectiveness of these media is obtained only after considerable experience with them. Only those media that inhibit the growth of most strains of *B. coli* should be used; the Conradi-Drigalski and Endo media are not satisfactory for the urine after incubation.

The manner of inoculating the plates after incubation of the urine-broth mixture is very important. Often there is an enormous multiplication of both typhoid bacilli and another bacillus that grows on the eosin brilliant-green plate; hence, unless the material is greatly diluted, the typhoid colonies are apt to be buried under a confluent growth of the latter organism.

As a routine procedure, we prepare from the incubated mixture dilutions as follows: 1:10, 1:100, 1:1000, 1:10,000, 1:100,000 and 1:1,000,000. Our saline solution is sterilized in 9 c.c. amounts, so these dilutions are quickly made. We then inoculate 1 loop of the 1:1,000,000, 1:10,000 and 1:100 dilution, and 1 loop of the original, each on a quadrant of the eosin brilliant-green plate. In this way, isolated colonies are always obtained on at least one of the quadrants.

Instead of preparing the dilutions described above, the inoculation of the incubated urine-broth mixture may be performed in the way

that is practiced in Japan for feces. The bottom of the eosin brilliant-green plate is ruled off with a wax pencil in the manner indicated in Figure 1. A loopful of the incubated urine-broth mixture is spread uniformly over the areas A and B. Without heating the loop, material is streaked from the base line of A over the surface of C, and from the base line of B over the area D. Then the loop is streaked over the area E, just touching the material in C at each stroke, and over F, first touching the material in D; in like manner from E over G, and from F over H. In this way an extreme dilution of the material is secured for the lower half of the plate.

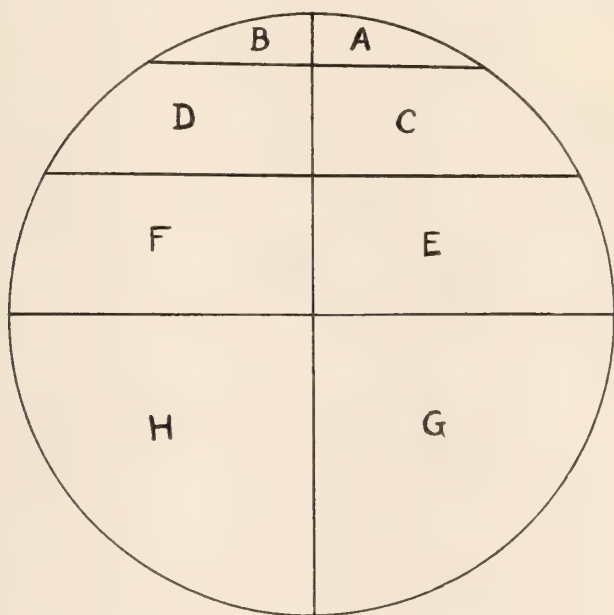


Fig. 1.—Bottom of eosin brilliant-green plate, ruled off for inoculation for incubated urine-broth mixture.

The first series of tests comprised 101 specimens of urine from typhoid patients, convalescents, and suspected typhoids at the Hoffman Island hospital. The urine was collected usually between 6 a. m. and 8 a. m., and was delivered at the laboratory at about 11:30 a. m. No attempt was made to sterilize the meatus of the urethra, nor was the first portion of urine that was passed discarded in order to reduce the amount of extraneous bacterial contamination. None of the specimens was obtained by catheterization.



Upon arrival at the laboratory, the urine was shaken and two large loopfuls were inoculated upon the surface of an eosin brilliant-green plate. About half as much broth as there was urine in the bottle was then added, and the mixture was incubated over night. The urine was not measured, the broth being added until it seemed about the right amount, as judged roughly by the eye. The amount of urine used for the test was usually about 2 or 3 ounces. After 18 to 24 hours' incubation, dilutions were made and an eosin brilliant-green plate was inoculated in the manner already described. If the plate inoculated directly with the urine showed typhoid colonies on the following day, it would, of course, be unnecessary, as a routine procedure, to make

TABLE 1

RESULTS OF TESTS ON URINE AND URINE-BROTH INOCULATED ON EOSIN BRILLIANT-GREEN PLATE FOR ISOLATION OF TYPHOID BACILLI

Urine Plated Directly	Urine Broth Incubated for 18 to 24 Hours and then Plated
+++	+++
—	+
+	+++
++++	+++
++++	+++
+	+++
++++	+++
++	++
—	+
++	++
++	++
+	++
+	+
++++	++
+	—
++	+++
—	+
++	++

+++ = numerous typhoid colonies.  
 ++ = fairly numerous typhoid colonies.  
 + = few typhoid colonies.  
 — = no typhoid colonies.

inoculations from the incubated urine-broth mixture; but in order to compare the results of the two methods, this was done in every instance in these tests. In Table 1, the samples of urine that were negative by both tests are omitted.

It is seen from Table 1 that 3 of the 19 positive urines were negative after direct plating, and positive after incubation with broth; in only 1 instance was the typhoid bacillus overgrown by other bacteria during the incubation of the urine with broth.

The second series of tests included 173 specimens of urine from 10 patients known to have typhoid fever. The specimens were collected without any attempt at the prevention of contaminations, at various stages of the disease and during convalescence, up to the time of discharge. Two loopfuls of the urine were inoculated directly upon an eosin brilliant-green plate. Ten c.c. amounts of the urine were pipetted into several test tubes; one tube was incubated without further addition and to each of the others were added 5 c.c. of broth, 3% dextrose in distilled water, or 5 c.c. of 0.6% sodium-chlorid solution, distilled water. After incubation, dilutions of the contents of the different tubes were prepared in the same manner for each, and eosin brilliant-green plates were inoculated as described in connection with Table 1. The results of these comparative tests are recorded in Table 2.

The results of the 161 tests shown in Table 2, in which the urines were incubated with broth, demonstrate 25 specimens positive on direct plating; 56 positive after incubation with broth; 2 positive on direct plating and negative after incubation with broth; 33 positive after incubation with broth and negative on direct plating, and 23 positive by both tests. Thus it is seen that out of a total of 58 positive urines, 33, or considerably more than half, would have been missed if the urines had been inoculated directly upon plates. For the sake of convenience and uniformity, only 10 c.c. amounts of urine were used in these tests; it seems probable that if larger amounts had been employed, still more positive results would have been obtained.

Broth was not added to 12 of the urines, but they were tested after enrichment, in the manner indicated in Table 2. The examination of all urines by the different methods yielded 74 positives out of 173 specimens. The comparative results from direct plating and from plating after enrichment showed 72 specimens positive after enrichment; 30 positive on direct plating; 28 positive by both methods; 2 positive on direct plating and negative after enrichment, and 44 positive after enrichment and negative on direct plating. Hence, of the 74 positive urines, 44 would have been overlooked if the urines had been inoculated directly on plates without enrichment.

Table 2 indicates that better results are obtained by incubating the urine plus broth than by incubating the urine alone; of 148 such comparative tests, 14 specimens of urine incubated with broth, positive, and alone, negative; 5 specimens incubated with broth, negative and alone, positive; 37 specimens of urine incubated positive by both tests, and 92 specimens incubated negative by both tests.

TABLE 2

RESULTS OF TESTS ON URINE INOCULATED ON EOSIN BRILLIANT-GREEN PLATE FOR ISOLATION OF TYPHOID BACILLI

Patient	Directly	After Incubation with				
		Broth	Dextrose Solution	Urine Alone	Sodium Chlorid Solution	Distilled Water
B. ....	++ ++ +++ — — — + ++ — — — — — — — —	+ + + ++ ++ — — + + + ++ + + — — — — — — —	..... ++ ++ ++ — ++ ..... ++ + — — — — — — —	..... ++ ++ ++ — ++ ..... ++ + — — — — — — —		
And. ....	— — — — — —	+++ — — — — —	..... — — — — —	..... — — — — —		
Per. ....	— — — — — — — — — —	— — — — + ++ — — — —	— — — — ++ ..... — — — —	— — — — — ..... — — — —		
San. ....	— — — — — —	+++ — — — — —	..... — ..... ..... ..... —	..... — ..... ..... ..... —		
Vit. ....	— — — — — — — — — —	+ + + ++ — — — — + — — —	..... — ..... ..... — — — — — —	..... ++ ..... ..... — — — — — —		
P. ....	— — — —	— — — —	..... — — —	..... — — —		

TABLE 2—Continued

RESULTS OF TESTS ON URINE INOCULATED ON EOSIN BRILLIANT-GREEN PLATE FOR ISOLATION OF TYPHOID BACILLI

[illegible]

TABLE 2.—Continued

RESULTS OF TESTS ON URINE INOCULATED ON EOSIN BRILLIANT-GREEN PLATE FOR ISOLATION OF TYPHOID BACILLI

Patient	Directly	After Incubation with				
		Broth	Dextrose Solution	Urine Alone	Sodium-Chlorid Solution	Distilled Water
M.....	—	—	.....	—	—	
	—	—	.....	—	—	
	—	—	.....	—	—	
	—	—	.....	—	—	
	—	—	.....	—	—	
G. ....	—	.....	.....	—		
	—	—	.....	—		
	—	—	.....	—		
	—	—	.....	—		
	—	++	.....	—		
	—	+	.....	—		
	—	.....	++++	+++		
	—	.....	++++	—		
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	—	++++	.....	++++	++++	+
	—	++++	.....	++++	++++	++
	+++	++++	.....	++++	++++	
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S. ....	—	.....	.....	++		
	—	++	.....	++		
	—	+++	.....	++		
	+++	+++	.....	++		
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	+++	—	+++	+++		
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	—	.....	—	—	—	++
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	—	—	.....	—	—	
	—	+++	.....	—	+++	
	—	+++	.....	+++	+++	
	—	—	.....	—	.....	
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	—	—	.....	—	—	

+++ = numerous typhoid colonies.  
 ++ = fairly numerous typhoid colonies.  
 + = few typhoid colonies.  
 — = no typhoid colonies.



Only 68 comparative tests were made with urine plus broth and urine plus dextrose solution. The results, though not conclusive, indicate that the broth mixture is slightly better. Five specimens of urine were positive with broth and negative with dextrose solution; 3 specimens were negative with broth and positive with dextrose solution; by both tests, 13 were positive, and 41 were negative.

Of 68 comparative tests, made between urines diluted with broth, and urines diluted with sodium-chlorid solution, 1 specimen incubated with broth, negative and with sodium-chlorid solution, positive; no specimen incubated with broth, positive and with sodium-chlorid solution, negative; by both tests, 21 specimens of urine were positive and 46 were negative.

A few comparative tests were carried out with dextrose broth instead of plain nutrient broth and with sterile distilled water in place of the sodium-chlorid solution, but not in sufficient number to furnish evidence as to the value of the methods.

#### SUMMARY

To isolate typhoid bacilli from urine not collected under aseptic precautions, streak 2 or 3 large loops of the urine over the surface of an Endo plate or preferably an eosin brilliant-green agar plate; add to the urine approximately one half its volume of nutrient broth and incubate the mixture over night. If the plate inoculated with the urine directly is negative the following morning, prepare dilutions of the incubated mixture of urine and broth and inoculate them on an eosin brilliant-green agar plate. Other special media that inhibit the growth of most strains of *B. coli*, while allowing the development of *B. typhosus*, could probably be substituted for the eosin brilliant-green agar, if one is not familiar with the latter medium. If the typhoid bacilli are present in sufficiently large numbers to yield colonies on the plate inoculated the first day, the incubated urine-broth mixture is, of course, discarded.

Sterilized bottles should be furnished for transporting the urine to the laboratory, and boiled urinals should be used for its collection, when practicable.

The method offers the advantage of subjecting a large amount of the urine to examination, with very little manipulation or loss of time. By its use it is believed that considerably higher percentages of positive results will be obtained in the routine examination of urines for typhoid bacilli than by the methods usually employed.

## RESULTS OF BLOOD CULTURES IN RHEUMATOID ARTHRITIS \*

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During the past 2 years we have made bacterial cultures from the blood of a large number of patients representing a wide variety of clinical conditions. We wish to present here a summary of the results in those cases in which acute or chronic involvement of the joints and adjacent tissues were the predominant or prominent clinical features. The literature contains few reports of this character. Rosenow<sup>1</sup> reports the isolation of a nonhemolytic streptococcus from the blood in 5 of 9 cases of rheumatic fever during the height of the attack, and occasionally in cases of proliferating osteoarthritis, arthritis deformans. By making cultures directly from the glands draining the involved joints in 54 cases, he isolated a modified *Strep. viridans* from 32 cases, staphylococcus in 5 cases, *B. mucosus* in 3 cases, *B. welchii* in 14 cases, and a diphtheroid bacillus in 5 cases. *M. catarrhalis* and the gonococcus were isolated once each. He found organisms in the joint fluid in a number of cases.

The method we used for blood cultures was essentially that of Rosenow. Fifteen c.c. or more of blood were drawn directly into a flask containing a sterile 0.5% solution of sodium citrate in distilled water. In  $\frac{1}{2}$ -2 hours, hemolysis being complete, the entire flask was centrifuged at 3000 revolutions per minute for 20-30 minutes. The supernatant fluid was drawn off and the sediment, consisting of leukocytes, stroma of red corpuscles and whatever bacteria were present, was rewashed with sterile water and centrifuged to free it further from hemoglobin. This sediment was then planted in various culture media under varying conditions of oxygen tension. Occasionally organisms developed in the glucose-ascitic broth, but more frequently minute colonies of streptococci were found in the deeper portions of tall shake-cultures in glucose-ascitic agar, where partial anaerobic conditions were present. Frequently the colonies would be visible as minute whitish specks in 2 or 3 days, but very often they developed

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<sup>1</sup> Jour. Am. Med. Assn., 1914, 63, p. 905.

more slowly. We often found streptococci by staining a smear from the sediment in the depths of agar-shake tubes which in 10 days of incubation had developed no visible colonies.

We make no attempt in this report to differentiate the types of conditions by using the terms osteoarthritis, proliferating arthritis, par-arthritis, etc. For convenience we have grouped the cases as acute and chronic, although these evidently are arbitrary terms, and the line cannot be sharply drawn which separates the groups. Cases of relatively short duration, with acute pain, fever, leukocytosis, etc., were considered acute. Those of longer standing with less pain and with little or no elevation of temperature we classed as chronic. Obviously as the one shades into the other a satisfactory differentiation is difficult.

Following are detailed histories of a few representative cases of different types:

1.—Merchant, aged 31. Attacks of inflammatory rheumatism 7 and 4 years previous, with apparently good recovery. For past 10 weeks an irregular intermittent temperature up to 104 F.; general weakness and pain in all the large joints, especially the hips.

The liver and spleen were much enlarged. There was tenderness in the region of the gallbladder. The heart was enlarged to the left and had a diffuse impact at the apex. There was a low pitched systolic murmur at the apex. Pulse was irregular in rate, 60-120. Blood pressure 125. Hemoglobin 60 per cent., whites 6600, polymorphonuclears 88 per cent.

Enlarged joints were extremely painful on pressure or motion; they showed no local redness but had an increased local temperature.

Diagnosis showed acute arthritis and acute mitral endocarditis.

The blood culture gave a gram-positive diplococcus which did not cause hemolysis nor produce a noticeable greenish tint on blood agar plates. Chain formation was not noticeable at first but after several generations of subcultures it became marked. This organism was classified as a nonhemolytic streptococcus.

A half-grown rabbit injected intravenously with the growth from the surface of a blood agar slant died within 48 hours. The mitral valve contained hemorrhagic points. Both hip joints were congested and contained increased fluid. The injected organism was recovered from the heart's blood and from the joint fluid.

2.—Man, aged 45. Complained of pain and stiffness in lumbar region, in right leg and neck. These points were very sensitive to pressure. Duration was 2 years.

The blood picture was normal. The Wassermann test was negative.

Roentgen rays showed a fusion of the vertebrae and proliferation of bone in the cervical and lumbar regions.

Diagnosis showed chronic myositis and proliferating arthritis.

The blood culture gave a nonhemolytic, nongreen-producing streptococcus. Second culture 3 weeks later gave the same result.

Growth from 2 blood agar slants injected intravenously into a young rabbit. Four days later the rabbit had impairment of motion in the hind quarters; lost weight steadily, would not eat, and moved about only after persistent provocation. Was chloroformed on the 12th day after inoculation. The mus-

culature was pale and flabby throughout, the viscera were somewhat injected and a phlegmonous infiltrating abscess was present in the gluteal muscles. The lumbar vertebrae were studded with hemorrhagic points and around and between the vertebrae was a seropurulent fluid which contained numerous streptococci.

3.—Woman; pain and deformity of joints of the extremities with markedly impaired mobility. Examination showed extensive pyorrhea alveolaris with badly decayed teeth.

The blood culture gave *B. capsulatus*, a vaccine of which was given; dental repairs were made to remove, if possible, the source of infection. Following this the patient was entirely free from pain, and the swelling and deformity of the joints was slowly improved, with increased mobility.

A year later there were symptoms of recurrence of tenderness and swelling in the joints. A 2nd blood culture resulted in isolating organism similar to the 1st. Vaccine treatment again was followed by relief. Whether this was permanent is not known as the patient was lost from observation.

4.—Man, aged 30; 6 years ago, following an attack of grip, the patient had acute rheumatic fever which kept him in bed 10 weeks. A stiffness of the joints of the back and hips developed.

The entire lower portion of the vertebral column was fixed and immobile, a typical 'poker-spine.' The hip joints also were immobile. Patient was able to walk after a fashion, with the body bent forward at an angle of about 90 degrees, crossing the legs in front of each other at each step by flexion of the knees. He had slight pain in the affected region. General condition of health good. The tonsils were enlarged and contained masses of cheesy material.

The blood culture gave a nonhemolytic streptococcus.

The tonsils were removed. An autogenous vaccine was given, and the reaction following the 1st few injections of this consisted of an intense aching pain in the affected joints. On subsequent injections there was no reaction and the patient had complete freedom of pain. His active symptoms subsided, but as would be expected, the ankylosis was permanent.

5.—Woman, aged 64. Two years ago gradual stiffening with soreness and pain began in the joints of the hands. Later the elbows, shoulders and knees were involved progressively and at time of examination scarcely a joint in the entire body was free from the affection. The sterno-mastoid muscles became sore and contracted spasmodically, at short intervals continuously, causing extreme discomfort.

The blood culture gave a nonhemolytic streptococcus.

The tonsils were removed; they contained numerous streptococci throughout their structure.

Persistent vaccine treatment was carried out using the organism obtained from the blood. The patient was given strict rest and attention was given to diet and hygienic conditions. Marked improvement followed. The general soreness and pain in the joints disappeared.

6.—Man, aged 35. Had a streptococcal skin infection of 1 finger from which a hemolytic streptococcus was isolated. The entire hand became red, swollen and painful, with red streaks running up the arm. The epitrochlear and axillary nodes became swollen and painful. Local infection subsided following free drainage and continuous hot moist dressings. Almost immediately the knee joints became enlarged and tender to movement and pressure. This was followed by severe involvement of the vertebral articulations in the lumbar region with pain on movement and pressure. At the same time severe pain developed in the right hypochondrium, slight fever and marked jaundice. The urine contained much bile pigment.



Diagnosis showed local streptococcic infection followed by acute arthritis, spondylitis and cholecystitis or cholangitis.

The blood culture gave a long chained, hemolytic streptococcus.

Autogenous vaccine treatment was combined with internal administration of elixir of iron, quinin and strychnin. Recovery was steady. Six weeks after the treatment began all symptoms had disappeared. There was no recurrence.

7.—Woman, aged 30. For 12 or 15 months the joints of the hands, wrists, ankles and feet had been very sensitive to pressure and movement. Joints were becoming progressively larger and limited as to motion. The involvement was so general and so severe that she was obliged to give up all work. The temperature showed irregular elevations of about 1.4 F. Roentgen rays showed marked increase of osseous tissue about the ends of the bones adjacent to the involved joints.

There was a history of tonsillar trouble, but the tonsils had been removed soon after the onset of arthritis with no effect on its development.

The 1st 3 blood cultures were negative; the 4th, taken after massaging and manipulating the involved joints, gave a nonhemolytic streptococcus.

Vaccine was prepared and administered continuously for 3 months, a part of which time the patient was kept at rest and given syrup of iodid of iron. The joint tenderness disappeared, the progress of the disease stopped, and she resumed work. A year later the joint trouble again became active and she was obliged to give up work. Roentgen-ray examination of the mouth showed an abscess about the root of a dead tooth. The pus pocket at the root of the tooth was removed and cultures of the pus gave streptococci mixed with diphtheroid bacilli. Autogenous vaccine treatment was persistently carried out, using a mixture of these organisms. For 6 months the patient has been free from symptoms and has worked uninterruptedly. The numerous enlargements about the joints have gradually become smaller with increased mobility of the joints.

8.—Man, aged 46. For past 4 weeks patient had pain and swelling in almost every joint with a low, irregular fever, fine petechial hemorrhages, most numerous over the abdomen; slight cough.

Moist râles were over base of lungs. The heart was enlarged markedly to the left. Low systolic murmur at apex; 1st and 2nd heart sounds feeble. The spleen was enlarged; the liver palpable. Hemoglobin 73%. White cells 17,000, 90% polymorphonuclear. The urine was scanty, containing albumin, a few pus cells and numerous granular casts. The joints were sensitive to pressure and motion, but showed no redness nor enlargement.

Diagnosis showed acute ulcerative endocarditis, acute nephritis, and acute arthritis.

The blood culture gave a nonhemolytic streptococcus which grew in firm discrete colonies and produced slight greenish color on blood agar.

Growth from a 24-hour blood slant was injected intravenously into a young rabbit. The rabbit soon lost weight, refused food, heart rate became rapid, and the animal evidenced pain on motion. At necropsy 9 days after injection, the heart and skeletal muscles were found pale and flabby; the mitral valve was nodular and contained areas of hemorrhage; the kidneys were pale; the left knee joint was dry and reddened; the hip joint contained an increased amount of serofibrinous fluid. A streptococcus like the one described was isolated both from the joint fluid and from the heart valve.

The patient did not recover.

The results in the animals inoculated with the organisms from blood cultures illustrate strikingly the localization of infection in



tissues corresponding to those infected in the patient. It is as if these freshly isolated strains have a specific selective affinity, as claimed by Rosenow, for certain tissues.

The association of joint involvement with endocardial infection, nephritis, and in 2 of these cases with infection of the bile passages is also worthy of note.

Attention is called to the teeth and tonsils, as well as to so-called 'grip' and local skin infection as original portals of entry for infection, which after entry may manifest itself in a variety of forms.

The results of autogenous vaccine treatment combined with tonics and hygienic measures seem good. An exception must be made in regard to cases with endocardial lesions in which in our experience autogenous vaccines are of no value.

Since detailed histories of all cases of joint infection in which we made cultures from the blood would be largely repetitions of the main points illustrated by the cases described, and would make this report voluminous, we have summarized them in Tables 1 and 2.

TABLE 1  
A TOTAL OF 40 CASES OF ACUTE ARTHRITIS

Case	Duration	Probable Source of Infection	Accompanying Conditions	Results*	Remarks
1	6 days	.....	Myositis.....	Streptococci	
2	4 weeks	Bad teeth.....	.....	Streptococci	
3	3 weeks	Pneumonia.....	.....	Negative	
4	7 days	Grip.....	Endocarditis.....	Streptococci	
5	6 days	.....	Myositis.....	Negative	
6	3 weeks	.....	Endocarditis.....	Negative	
7	3 months	Teeth.....	.....	Diphtheroid bacillus	Vaccine with apparent benefit
8	3 weeks	Grip.....	Carditis.....	Negative	
9	3 weeks	Skin infection.....	.....	Streptococci aureus	
10	4 weeks	Tonsils.....	.....	Streptococci.....	Vaccine with apparent benefit
11	4 months	Pneumonia.....	Myositis.....	B. mucosus capsulatus	Vaccine with little benefit, if any
12	4 weeks	Otitis media.....	Neuritis.....	S. hemolyticus	
13	3 weeks	Acute tonsillitis.....	Myositis.....	B. mucosus capsulatus	
14	5 weeks	Pyorrhea.....	.....	Negative	
15	4 weeks	.....	.....	Streptococci.....	Vaccine with no benefit
16	6 months	.....	Nephritis.....	Streptococci	
17	4 months	.....	Involvement of facial nerve	Diphtheroid	
18	7 weeks	.....	Rheumatism.....	Negative	
19	5 months	.....	.....	Streptococci.....	Vaccine with apparent benefit
20	6 months	Gonorrhea.....	Gonorrheal salpingitis	Negative	
4 cases		Described in detail above.....		Streptococci all 4	See case reports
16 cases		Grouped.....		2 streptococci, 14 negative	

\* Results of 40 cases of acute arthritis: nonhemolytic streptococci, 13; hemolytic streptococci, 1; B. mucosus capsulatus, 2; diphtheroid bacillus, 2; staphylococcus aureus, 1; negative, 21.

TABLE 2  
A TOTAL OF 83 CASES OF CHRONIC ARTHRITIS

Case	Duration	Probable Source of Infection	Accompanying Conditions	Results*	Remarks
1	.....	Bad tonsils and teeth	Myositis.....	Streptococcus....	Vaccine with apparent benefit
2	.....	Pyorrhea.....	Nephritis.....	Streptococcus....	Vaccine with apparent benefit
3	.....	Tonsils.....	Endocarditis....	Negative	
4	6 years	Grip.....	.....	Streptococcus	
5	1 year	Sinus infection....	.....	Negative	
6	Years	Pyorrhea.....	Myositis.....	Streptococcus....	Vaccine with benefit
7	.....	Chronic nasopharyngitis	Sinus infection chronic	Streptococcus	
8	4 years	Specific urethritis	Myositis.....	Negative	
9	12 years	.....	Nephritis	Streptococcus....	Vaccine with some benefit
10	18 months	.....	Myositis	Streptococcus....	Vaccine with apparent benefit
11	2 years	.....	Chronic posterior urethritis	Negative	
12	Years	Frequent attacks of tonsillitis	Myositis.....	Streptococcus....	Vaccine with benefit
13	1 year	Tonsils.....	Neuritis.....	Negative	
14	5 years	.....	.....	B. mucosus capsulatus	Vaccine with benefit
15	4 months	Pyorrhea.....	Extreme muscular soreness	Streptococcus....	Vaccine with apparent benefit
16	15 years	Tonsils.....	Recurrent myositis	Negative	
17	1 month	Pyorrhea.....	Myositis.....	Streptococcus	
18	3 years	.....	.....	2 cultures of streptococcus in both	
19	4 years	.....	Paralysis and neuritis	Negative	
20	6 years	.....	Sciatic neuritis, lumbago	Streptococcus....	Vaccine with apparent benefit
21	2 years	.....	.....	3 cultures B. mucosus capsulatus in all	Vaccine with marked benefit
22	14 years	Otitis media.....	Suspected tuberculosis	Diphtheroid	
23	Several years	Tonsils.....	.....	Streptococcus....	Vaccine with marked benefit
24	5 years	Tonsils.....	Marked ankylosis	4 cultures 1 streptococci	Vaccine, no benefit
25	4 months	.....	.....	Diphtheroid	
26	5 years	.....	.....	Diphtheroid.....	Vaccine with benefit
27	7 years	Puerperal infection	Sciatic neuritis....	Negative	
28	14 years	.....	Extensive ankylosis	3 cultures, all negative	
29	4 months	.....	.....	Diplococcus, which later grew as a diphtheroid	Vaccine with benefit
30	Several years	.....	.....	4 cultures, all negative	
4 cases		Described in detail above.....	.....	1 B. mucosus, 3 streptococci	See case reports
49 cases		Grouped.....	.....	2 streptococci, 47 negative	

\* Results of 83 cases of chronic arthritis: nonhemolytic streptococci, 18; B. mucosus 3; diphtheroid bacilli, 3; unidentified organisms, 1; negative cultures, 58.

## DISCUSSION

From these results we believe that frequently, though not regularly, in arthritis, pararthritis, and myositis, organisms may be isolated from the blood stream by improved cultural methods. This is possible more frequently in the acute stages when the patient is in a febrile condition, and less frequently in the subacute and chronic stages of the infection. This we assume is because in the chronic stages the organisms are more deeply embedded in the affected tissues and occur less frequently free in the blood stream. As seen from the cases described above, streptococci were the organisms most frequently cultivated, and these correspond in type to those isolated by other observers from joints, glands, and tonsils of arthritic patients. In chronic cases we have been able to cultivate streptococci from a few drops of fluid aspirated from the joints or from neighboring involved bony parts in 6 of 10 cases.

We have found other organisms than streptococci in a small percentage of cases as follows: diphtheroids 4%, *B. mucosus* 4%, staphylococci 0.8%. These findings corroborate those of other observers who believe that occasionally the joint structures are infected by diphtheroids and *B. mucosus* as well as by streptococci, staphylococci, pneumococci, gonococci, typhoid bacilli, etc. Rosenow<sup>2</sup> states that the pathogenic property of the diphtheroid group of bacilli is similar to that of the streptococci and, as quoted, has isolated diphtheroids frequently from the adjacent lymph nodes in chronic arthritis. Our experience with this group of organisms in cases of various types leads us to the belief that while many strains of diphtheroid bacilli occur about the body as apparently harmless saprophytes, yet certain strains are unquestionably pathogenic, which is likewise true concerning the staphylococcus and streptococcus groups. Regarding the *B. mucosus*, Rosenow also isolated it from cases of chronic arthritis, and Dick<sup>3</sup> has reported in detail cases of chronic arthritis as caused by this bacillus. In one of our cases this organism was cultivated from the blood on 3 successive cultures at intervals of several months.

We do not hold that the cultivation of the organisms from the blood or joints in these cases is definite proof of their causal relationship to the rheumatoid arthritis. There is the possibility present that in some instances the organism isolated was associated with some concurrent infection. Yet the isolation of these organisms cannot be

<sup>2</sup> Surg., Gynec. and Obst., 1915, 20, p. 404.

<sup>3</sup> Jour. Infect. Dis., 1914, 14, p. 176. Jour. Am. Med. Assn., 1917, 68, p. 622.

overlooked in summing up the evidence regarding the etiology of arthritis.

The fact that the patients, from whom diphtheroids and *B. capsulatus* were isolated, improved under autogenous vaccine treatment still further suggests the causal relationship of the organism to the condition in these cases.

The results of autogenous vaccine treatment when consistently carried out were for the most part gratifying, though there were cases as noted where little or no improvement was evident. In making a statement regarding results of vaccine treatment we have not lost sight of the tendency of infection to spontaneous improvement or recovery, nor of the fact that in most of these cases improved hygienic conditions, rest and tonics were also factors. However, there is but little permanent improvement to be secured in chronic progressive arthritis by these measures alone, yet a number of our cases showed marked permanent improvement when these measures were combined with autogenous vaccines.

#### CONCLUSIONS

Streptococci may frequently be cultivated from the blood stream in rheumatoid arthritis.

There is evidence that the so-called diphtheroid bacilli and *B. mucosus* occasionally cause infection of the joint structures.

Autogenous vaccines combined with other measures have been followed by marked improvement in cases of arthritis of various types.

## CORRELATIONS IN THE COLON-AEROGENES GROUP\*

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Recent papers by Rogers and Clark and their associates, and those of Levine and others, have done much to create a new interest in the classification of organisms in the colon-aerogenes group. The correlation of specific characters of members of this group with their habitat has been sought for some time on account of its sanitary significance, but until recently no progress has been made which has attracted any considerable degree of attention.

Harden<sup>1</sup> and MacConkey<sup>2</sup> have discussed the action of *B. cloacae* and *B. [lactis] aerogenes* on the sugars, particularly glucose, and noted the infrequency with which these 2 organisms were met in fecal matter. Both of these investigators observed the extensive utilization of glucose by these organisms as compared with the colon bacillus proper, and stated that in most instances the glucose was completely exhausted from the culture medium by *B. cloacae* and *B. [lactis] aerogenes*, in distinction from the other members of the entire gas-forming group. They also pointed out the correlation of this property with the Voges and Proskauer reaction, which none of the other organisms under observation exhibited. In fact the Voges and Proskauer reaction was thought at this time to be an easy means of distinguishing between the members of the group which are of fecal and those which are of non-fecal origin.

Rivas<sup>3</sup> in discussing the so-called 'saccharolytic group' of sugar-fermenting bacteria, which includes *B. cloacae* and *B. [lactis] aerogenes*, states that since the colon bacillus is not a member of this group it should not be employed for the removal of muscle sugar from meat extract, in the preparation of nutrient broth, but that 1 of the organisms which cause rapid and complete decomposition of glucose and glucose-like sugars, namely the saccharolytic group, should be used. Kendall, Day and Walker<sup>4</sup> have likewise shown that the colon bacillus differs in certain important respects from *B. cloacae*, aside from gelatin liquefaction. *B. cloacae* ferments glucose broth, giving a CO<sub>2</sub>:H ratio of 2:1, as was first shown by Smith.<sup>5</sup> Furthermore, members of the *cloacae* group decompose glucose rapidly, the carbohydrate disappearing from the culture medium within 24 hours. The colon bacillus, under similar con-

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<sup>1</sup> Jour. Hyg., 1905, 5; p. 488. Proc. Roy. Soc., 1906, B, 77, p. 424. Ibid., 1906, B, 77, p. 399.

<sup>2</sup> Jour. Hyg., 1906, 6, p. 385. Ibid., 1909, 9, p. 86.

<sup>3</sup> Jour. Med. Research, 1908, 18, p. 81.

<sup>4</sup> Jour. Am. Chem. Soc., 1913, 35, p. 1227.

<sup>5</sup> Wilder Quarter Century Book, 1893, p. 212.



ditions, produces a much larger amount of acid than *B. cloacae*, the acid of the former being of sufficient strength to cause complete cessation of growth.

These suggestions received little attention until Rogers, Clark, and Davis<sup>6</sup> by the methods of exact gas analysis made a critical study of the colon-aerogenes group and found that in general 2 subgroups of organisms existed, namely the 'high ratio' or cloacae division, and the 'low ratio' or colon subgroup. These could be subdivided further into 6 lower divisions, as was pointed out by Rogers, Clark, and Evans.<sup>7</sup> These same investigators<sup>8</sup> when studying the bacteria of bovine feces, observed that only 1 of 150 strains of gas-formers gave a gas ratio which identified it with the high ratio organisms.

Clark and Lubs<sup>9</sup> showed that the bacteria of the high and low ratio groups could be distinguished from each other readily by the hydrogen-ion concentration<sup>10</sup> resulting from the fermentation of glucose in a medium of the following composition:

Witte's peptone.....	5 gm.
Glucose .....	5 gm.
K <sub>2</sub> HPO <sub>4</sub> .....	5 gm.
Distilled water.....	1000 c.c.

Levine<sup>11</sup> in his study of the newly proposed methyl-red test observed that the organisms which gave no red coloration in Clark and Lubs medium were also those which were positive by the Voges and Proskauer test, and that none of the methyl-red positive organisms gave the Voges and Proskauer reaction.

Levine's observations were apparently confirmed by Hulton<sup>12</sup> and Greenfield,<sup>13</sup> who regard these correlations as being of much sanitary significance.

The work of Prescott and his pupils,<sup>14</sup> Papastiriu,<sup>15</sup> Metcalf, Winslow and Walker,<sup>16</sup> Fromme,<sup>17</sup> Rogers, Clark, and Evans,<sup>7</sup> Johnson,<sup>18</sup> and others has indicated that gas-forming bacteria of the colon group type are found in nature in places where there has in all probability been no recent fecal contamination.

In 1914 it was decided by one of us to make an exhaustive study of such organisms, particularly those which are found in the soil, and to endeavor to classify them. The work extended over 2 years, and while it is far from complete in certain respects, the results may prove to be of some assistance to others who are engaged in similar studies.

<sup>6</sup> Jour. Infect. Dis., 1914, 14, p. 411.

<sup>7</sup> Jour. Infect. Dis., 1914, 15, p. 99.

<sup>8</sup> Ibid., 1915, 17, p. 137.

<sup>9</sup> Ibid., p. 160.

<sup>10</sup> The hydrogen-ion concentration was determined after 3-5 days' incubation at 30 C., methyl red being used as the indicator.

<sup>11</sup> Jour. Bact., 1916, I, p. 153. Jour. Infect. Dis., 1916, 18, p. 358.

<sup>12</sup> Ibid., 19, p. 606.

<sup>13</sup> Ibid., p. 647.

<sup>14</sup> Biological Studies by Pupils of W. T. Sedgwick, Boston, 1906, p. 208.

<sup>15</sup> Arch. f. Hyg., 1902, 41, p. 439.

<sup>16</sup> Science, 1905, 26, p. 797.

<sup>17</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1910, 65, p. 251. Ibid., 1913, 74, p. 74.

<sup>18</sup> Abstr., Jour. Bact., 1916, 1, p. 96.

## METHODS OF TITRATION

The titration methods of various workers were first investigated. The absence of uniformity in the method of procedure became very apparent, and it is not surprising that results of different investigators should be in some instances so conflicting. Most of the titration experiments have been conducted on both fresh media and culture fluids of different ages.

From the available data it was seen that there is a tendency toward the hot titration and the subtraction of a blank titration, in order to determine the amount of acid formed. Is this the correct method? Before attempting to answer this question let us first consider the significance of the term 'acidity.'

If the hydrogen-ion concentration in a culture is a growth function of the ability of the organism to resist its own acid, and is due to no other cause, we would expect to obtain the same acidity with all sugars which a given organism can ferment with the production of acid. This is seldom observed in practice, however, for instances of high acidity in one sugar and low in another are common. It is recognized that a sufficient amount of acid will ultimately cause the death of an organism, or complete cessation of all its activities, but unless death does ensue it is difficult to believe that the whole organism is affected in such a way that no more acid can be produced. Rather would the cause of the failure to produce any more acid be looked for in the inhibiting effect of the end-product on the particular biologic mechanism which brings its formation about. Present day views on the action of enzymes would favor the idea that an equilibrium would be reached. Such equilibrium may or may not coincide with the acid death point of Holman.<sup>19</sup>

This view is not materially different from that enunciated by Hopkins and Lang,<sup>20</sup> but it is evident from their subsequent work that they disregarded it.

Finally, the principles of physical chemistry must be respected, as was clearly stated by Clark and Lubs, and by Clark alone. The titration of media containing protein substances, meat extract, and inorganic salts, together with the unknown substances which are elaborated by bacteria, is a procedure which can in no way give the true idea of conditions which actually govern the acid-production, or hydrogen-

<sup>19</sup> Jour. Infect. Dis., 1914, 15, p. 227.

<sup>20</sup> Ibid., p. 63.

ion concentration, on account of the buffer effect of the substances present.

An example of the buffer effect in 2 media which were often used in this research is given in the following figures:

- |     |                                |                                      |
|-----|--------------------------------|--------------------------------------|
| I.  | 1000 c.c. water                |                                      |
|     | 5 gm. Witte's peptone          |                                      |
|     | 5 gm. $K_2HPO_4$               | Phenolphthalein acidity 1.0%. Normal |
|     | 5 gm. glucose                  |                                      |
| II. | 1000 c.c. water                | (Chief medium of this research)      |
|     | 10 gm. Witte's peptone         | Phenolphthalein acidity 2.1%. Normal |
|     | 10 gm. glucose                 |                                      |
|     | 4 gm. Liebig's extract of beef |                                      |
|     | 5 gm. $K_2HPO_4$               |                                      |

Both of these media are neutral to litmus, and therefore have a hydrogen-ion concentration of about  $10^{-7}$  N. According to Clark and Lubs, the same organism will reach about the same hydrogen-ion concentration in each, and yet the titration figures when these 2 media were inoculated with a laboratory strain of the colon bacillus were: acidity 2.3% normal and acidity 5.4% normal, respectively.

Thus, in cases where the acidity in the 1st medium may show small differences between different cultures, the buffer action of a 2nd medium might be so used as to magnify the differences, instead of affording a more refined method of titration. It is known, however, that titration figures give only relative, and not true values.

In order to determine whether the titration method, as commonly practiced, is the correct one, answers to the following questions were sought:

1. What is the effect of initial acidity on terminal acidity?
2. Shall a blank titration be subtracted from the terminal acidity?
3. What is the effect of surface exposure to air during cultivation?
4. Shall the carbon dioxide be boiled off?
5. Shall the titration be made in a hot or in a cold medium?

#### INFLUENCE OF INITIAL ON TERMINAL ACIDITY

The fallacy of adjusting the reaction of media has been shown by Clark;<sup>21</sup> but the question arises, "What would be the effect on the growth of an organism of a medium which does not correspond with what we have been pleased to call correct acidity?" If the acidity is shown to be too high it might be expected that either growth could not

<sup>21</sup> Jour. Infect. Dis., 1915, 17, p. 109.

take place or the medium would be so interfered with that the usual results could not be obtained. This point may be further elucidated by determining whether acid-production is the result of growth or of the metabolism of cells in the living state, but which are not actively reproducing.

To study this point 40 c.c. portions of broth containing 1.3% glucose, 0.02%  $\text{Na}_2\text{HPO}_4$ , 0.4% meat extract, and 1.0% peptone were placed in 250 c.c. Erlenmeyer flasks and sterilized. Varying amounts of N/20 HCl were added, the flasks incubated for 24 hours to determine the sterility, and then inoculated with 0.5 c.c. portions of actively fermenting broth cultures of 2 different types. Table 1 is representative of several tests. After the first few tests direct microscopic counts of cells in the various flasks were made, in order to detect a direct relationship between acidity and numbers of bacteria.

TABLE 1  
EFFECT OF INITIAL ACIDITY ON TERMINAL ACIDITY AND THE TOTAL NUMBER OF  
BACTERIA PRESENT

Flask No.	Culture No.	Acid Added c.c. N	Initial Acidity % N	24-Hour Acidity % N	24-Hour Count Cells per c.c.	60-Hour Acidity % N	60-Hour Count Cells per c.c.	120-Hour Acidity % N	120-Hour Count Cells per c.c.
1	*	0.0	1.65	0.73		0.86		1.06	
2	*	2.5	1.83	1.56		1.35		1.41	
3	*	5.0	2.09	1.86		1.84		1.70	
4	*	7.5	2.30	2.17		1.96		1.90	
5	*	10.0	2.56	2.51		2.37		2.40	
6	23	0.0	1.45	1.46	25,500,000	0.55	640,000,000	0.10	47,200,000,000
7	23	2.5	1.51	1.76	58,500,000	1.20	900,000,000	0.10	73,200,000,000
8	23	5.0	1.56	1.81	73,500,000	0.48	1,540,000,000	0.10	74,100,000,000
9	23	7.5	2.15	2.14	58,800,000	0.56	1,000,000,000	0.10	9,570,000,000
10	23	10.0	2.22	2.36	5,180,000	2.03	300,000,000	1.52	7,750,000,000
11	43	0.0	0.80	4.09	36,700,000	4.10	370,000,000	4.30	9,200,000,000
12	43	2.5	1.20	3.58	61,200,000	3.46	440,000,000	3.55	5,680,000,000
13	43	8.0	1.70	†					
14	43	7.5	1.50	3.50	18,800,000	3.40	280,000,000	3.59	4,840,000,000
15	43	10.0	2.43	2.58	4,150,000	2.50	500,000,000	2.52	750,000,000
16	46	0.0	1.97	1.31	62,000,000	0.42	820,000,000	0.30	13,670,000,000
17	46	2.5	0.81	1.86	76,000,000	0.50	550,000,000	0.30	34,400,000,000
18	46	5.0	1.40	2.15	137,000,000	0.65	1,020,000,000	0.40	23,800,000,000
19	46	7.5	2.00	2.25	25,200,000	0.55	474,000,000	0.42	15,300,000,000
20	46	10.0	2.48	2.50	276,000	2.43	124,000,000	2.40	4,300,000,000

\* Uninoculated.

† Contaminated.

From Table 1 it appears evident that within reasonable limits the initial acidity of a medium does not have a very marked effect on the terminal acidity. Later work has shown that the Organisms 23 and 46 were of the high ratio type of Rogers, Clark, and Davis, while 43 was of the low ratio type. As to the high ratio organisms, it is seen that up to 2% of initial titrable acidity there is no difference in the final acidity. From the rate of growth it is apparent that in the neighborhood of 1.5% acidity the optimum conditions for growth are obtained, in so far as reaction is concerned. That mere numbers of cells do not

play an important part is seen by comparing the numbers of cells in Flasks 8 and 9, and 16 and 17 at the 120-hour period. It is also clear that even in the case of the largest amount of added acid there is actual growth and a reduction of the initial acidity, which might be expected ultimately to reach the same acidity as the corresponding unacidified culture.

With regard to low-ratio Organism 43, it is apparent that within certain limits this organism can reach its maximum acidity within 24 hours, and that after this time the reaction is constant, although growth actually does occur as is shown by the increased number of cells. It is shown further that within these limits of acidity practically the same final acidity has been reached. Too much weight must not be placed, therefore, on small differences of acidity.

From this and similar experiments not recorded here the 1st question may be answered as follows: Within the limits of rapid growth the initial acidity has very little or no effect on the terminal acidity.

#### SUBTRACTION OF INITIAL FROM FINAL ACIDITY

With reference to Question 2 it may be seen that to subtract the initial apparent acidity from the terminal acidity would be fallacious, and would show nothing of value if the results are to be treated statistically. Brown<sup>22</sup> believed that the amount of acid formed was the vital issue, for he says, "The amount of acid produced by the *B. coli* group in different carbohydrate media depends in a great part upon the initial reaction . . . The maximum acidity is the amount necessary to prevent further growth." If the amount is the really important point, Brown is correct, for the foregoing data show that the amount of change brought about by an organism is the function of the initial acidity; but when a number of organisms arrive at the same acidity ultimately, and when the problem is approached from different stand-points, it is evident that the common limiting value is the more important and more constant factor.

From Brown's own figures<sup>22</sup> and from Table 1 it may be stated definitely that blank titrations should not be subtracted to determine the acid-production, although this is contrary to the conclusion which Brown himself drew.

The slight change which is observed in the titrable acidity of uninoculated media may be due to the slowness of the buffer action of the medium.

<sup>22</sup> Jour. Infect. Dis., 1914, 15, p. 580.



## INFLUENCE OF SURFACE EXPOSURE ON FINAL ACIDITY

For some time we were led to believe that the area of liquid exposed to the air would play an important part in determining the final acidity, but later it was found that these conclusions were erroneous. Subsequent work which is not presented here, owing to the size of the tables involving over 1000 titrations, indicator tests, and Voges and Proskauer reactions, has enabled us to draw the following conclusions, in answer to Question 3: No great difference in the titrable acidity is observed when the bottle (large surface) and deep test tube methods of cultivation are employed. There is in some instances a greater tendency toward variability in the large surface method. The sugar utilization is usually more complete in test tubes than in bottles lying on their sides and exposing 30 sq.c. of surface of liquid.

INFLUENCE OF BOILING BEFORE TITRATION ON THE TITRATION RESULTS  
AND THE COMPARATIVE VALUES OF HOT AND  
COLD TITRATIONS

There has always been some discussion among bacteriologists as to the proper methods of determining titrable acidity. The chemist who would titrate a liquid using phenolphthalein as an indicator without first boiling off the dissolved  $\text{CO}_2$  would be looked on askance by his associates. But the chemical methods have often been transferred bodily without any question as to applicability, and today nearly all bacteriologists endeavor to get rid of  $\text{CO}_2$ . Most workers recognize the potential danger of driving off volatile acids or bases by boiling, some attempting to compromise by steaming the tubes, while others continue to boil because it is the official method of the American Public Health Association.

The solution of the problem involves the study of the influence of boiling on both uninoculated and inoculated (incubated) media. Here again it is necessary to determine the most efficient methods of procedure. First, the question arises: Which of the titrations gives the most accurate results, the hot or the cold method? In Table 2 the hot and cold titrations for Clark and Lubs' broth are given. In this test the medium was brought to the boiling point, but not boiled. The flasks marked 'cold' were heated, cooled, and titrated, while the 'hot' flasks were being heated. The heating process took the same length of time in each case.

TABLE 2  
ACIDITY OF UNINOCULATED CLARK AND LUBS' BROTH

Titration Number	Titrated Acidity	
	Hot	Cold
1.....	1.65	1.60
2.....	1.80	1.55
3.....	1.70	1.58
4.....	1.70	1.60
5.....	1.60	1.60
Average acidity.....	1.69	1.58

According to Table 2, the hot titration gives higher results than the cold, which fact was observed by Winslow and by Holman,<sup>19</sup> but which has either escaped other observers or has been ignored by them. The more or less popular notion that the hot titration is more accurate as to sensitiveness than the cold titration is disproved in this instance at least.

The higher figure in the hot titration as compared with the cold is probably due to the difference in the degree of ionization of the hot and cold media. The medium when neutral to phenolphthalein while hot becomes pink on cooling and colorless again on subsequent heating. The difference between the hot and cold titrations is probably a measure of the influence of heat on the ionization phenomena in this particular solution.

It may be argued that in making a titration the liquid should be boiled and then cooled. Such a practice would be both time-consuming and erroneous, for it will be shown that the boiling off of CO<sub>2</sub> introduces a very serious error. This is in confirmation of the statement of Anthony and Ekroth.<sup>23</sup>

TABLE 3  
A COMPARISON OF HOT AND COLD TITRATIONS OF BOILED CULTURES

Number	Minutes Boiled	Culture Numbers							
		368		G. F. 11		386		1080	
		Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold
1	0.0	1.16	0.95	2.99	2.81	0.88	0.49	2.28	1.97
2	0.25	1.06	1.07	3.00	2.81	0.92	0.49	2.40	1.97
3	0.5	1.20	0.99	3.00	2.82	0.93	0.68	2.25	1.96
4	1.0	1.24	1.11	2.99	2.76	0.94	0.67	2.25	1.93
5	2.0	1.25	1.30	2.93	2.77	1.06	0.82	2.23	1.98
6	5.0	1.27	1.43	2.94	2.71	1.35	1.14	2.11	1.97

The period of incubation was 5 days.

<sup>23</sup> Jour. Bact., 1916, I, p. 209.

The next point to determine experimentally is whether cultures should be titrated hot or cold. For this purpose several flask cultures were made, samples were taken, diluted, and boiled for the periods indicated. They were done in duplicate, in 2 sets, 1 set being titrated while hot and the other after allowing it to cool to the room temperature. A comparison of the averages is given in Table 3.

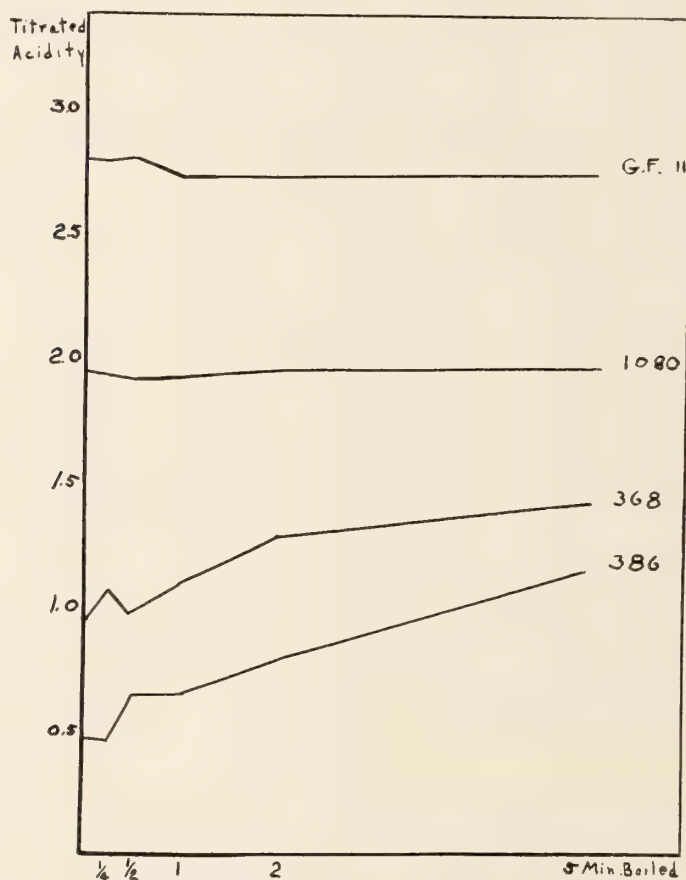


Fig. 1.—Effect of boiling on titrable acidity. Cold titrations.

Table 3 shows that boiled cultures have a tendency to show higher results when hot titrations are employed than when the liquid is allowed to cool to room temperature.

It also shows the effect on the acidity of boiling off  $\text{CO}_2$ , whether the titration is made hot or cold. This is made more evident graphi-

cally as is shown in Figure 1, where the results of the hot titrations are plotted.

It is difficult to draw any definite conclusions from the results. In general, they seem to show that no  $\text{CO}_2$  is removed by boiling, for otherwise one might expect a nearly uniform decrease of acidity for a short time at least. The apparent acidity of the high ratio organisms is increased, due perhaps to the driving off of a volatile base. The general trend of the curve does indicate that boiling or the mere addition of boiling water does not tend to make the results any more constant, but seems to introduce an error in the case of the high ratio cultures, and therefore should not be done. These experiments confirm Clark's statement<sup>21</sup> that the results of titrations are vitiated by the hot method.

On the other hand, the recent abstract of Noyes<sup>24</sup> stating that  $\text{CO}_2$  in distilled water will introduce grave errors cannot be accepted in the sense that he seems to have meant it. He says that distilled water or double-distilled water does not mean carbon-dioxid-free water, and further, " $\text{CO}_2$  in distilled water makes it possible to have a medium titrate plus 1.0% when it is really neutral or alkaline." It seems as though he must have been working with distilled water particularly rich in  $\text{CO}_2$  to arrive at such conclusions, but as his data have not yet been published, this is only a conjecture.

On completion of this preliminary work it was decided to cultivate the organisms in glucose-phosphate broth (the composition of which has been given), in bottles and in test tubes. The titrations were made in the cold, without boiling off  $\text{CO}_2$ , the culture fluid being diluted with cold distilled water which had been recently boiled. The period of incubation was 5 days at 37 C.

#### BIOMETRIC STUDIES OF THE GAS-FORMERS OCCURRING IN NATURE

*Preparation of Cultures.*—About 1000 samples of soils, leaves, twigs, flowers, bark of trees, berries, sand, snow, etc., were collected in places which were situated as far as possible from all sources of pollution. These samples were taken from isolated fields in Illinois, sand dunes in northern Indiana, mountain tops in Connecticut, and the uppermost points of the watershed of the New Haven Water Company. The possibility of fecal contamination on the watershed

<sup>24</sup> Jour. Bact., 1916, 1, p. 87.

and on the mountain tops is extremely remote. With very few exceptions there was no reason to believe that there was any recent fecal contamination of any of the samples from the Middle West. The exceptions mentioned and indeed any samples which were taken from places where the drainage was toward the point of sampling, are treated separately, thus necessitating 2 groups of bacteria, 1 set being designated as 'O. K.' from the sanitary standpoint, and the other marked 'Discards.' The classification study was made on the combined sets.

All of the samples were inoculated in glucose broth and incubated at 37 C. for 3 days. Any of the cultures which showed gas-production were plated. The plating was done originally on litmus glucose agar, but during the 2nd year it was found that the predominating gas-former in soil was not an acid-producer in these agar plates. The use of litmus was discontinued and it was found necessary to test the gas-production of representative agar colonies. Gas-forming cultures were plated out again and isolated colonies tested for gas-production, as before. This process was carried out a 3rd time, after which a well isolated colony was selected for the inoculation of the medium whose composition is given on p. 165 (II). Following an incubation period of 5 days at 37 C., the broth cultures were titrated.

Whenever there was any question as to the purity of any given culture the following method of replating was carried out: The culture material was vigorously shaken for 5 minutes in a large test tube containing broken glass and 5 c.c. of water, after which a loopful of the emulsion was transferred to a 2nd tube and the shaking repeated. Plates were poured from the 2nd tube. Glucose broth in Durham fermentation tubes was inoculated from 5 of the most varied colonies.

The morphology of the organism was studied on Gram-stained slides. With few exceptions the gas-producing organisms were Gram-negative, short rods having spores were never observed. In their microscopic appearances and in their general behavior they resembled the colon bacillus. The Gram-positive organisms that were met with only occasionally were spore-producers and relatively large, and it was evident that they were members of a widely different group.

*Biometric Data.*—Cultures were made in the glucose phosphate broth (II, p. 165) in Durham fermentation tubes and in 4-ounce glass bottles which gave a surface exposure of 30 sq.c. when laid on their sides. Approximately the same amount of liquid was placed in the tubes as in the bottles.

Titration was made as indicated.

Methyl red was added to the broth to determine on which side of the methyl red neutrality point the acidity lay.

Qualitatively sugar determinations were made at first with Fehling's solution, but a purple color was observed to develop in many cases which was in all probability due to the Voges and Proskauer<sup>25</sup> reaction, brought about by boiling with the strongly alkaline solution. Consequently Benedict's solution (2nd modification) was substituted and the troublesome purple color did not appear. Saccharose was inverted by boiling with a few drops of sulphuric acid for a half hour.

<sup>25</sup> Ztschr. f. Hyg., u. Infektionskrankh., 1898, 28, p. 20.



The value of the Voges and Proskauer reaction in its relation to this group of organisms was not appreciated until about half of them had been studied. It was tried with all of the sugar media and with some of the glycerin broth tubes. In glycerin media the reaction was present in a great many instances, but it was weak and uncertain and therefore discontinued. The reaction was obtained by adding 10 c.c. of 2-4% KOH to the sugar media and letting them stand over night.

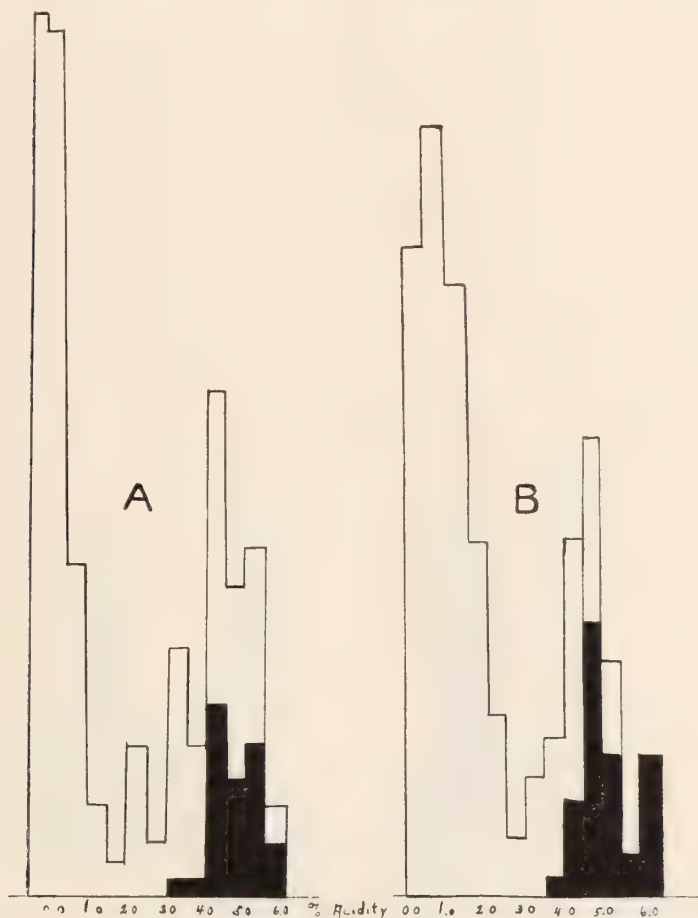


Fig. 2.—A. Glucose in bottles. Shaded area = low ratio cultures according to Clark and Lubs' methyl red test. B. Glucose in fermentation tubes. Shaded area = low ratio cultures according to Clark and Lubs' test.

Pigment-production was recorded in the standard color terms of the chart of the Society of American Bacteriologists. Owing to the large number of cultures and to the vast number of other tests, it was impossible to go into the subject of pigment-production as extensively as Rogers, Clark, and Evans.

The determination of gelatin liquefaction by the standard method was undertaken, but this procedure was abandoned as too uncertain, and the method proposed by MacConkey<sup>2</sup> was tried and found to be more satisfactory. This test is made by using 5% gelatin and incubating at 37 C., removing the tubes from time to time and cooling for several hours. In this way all liquefiers were

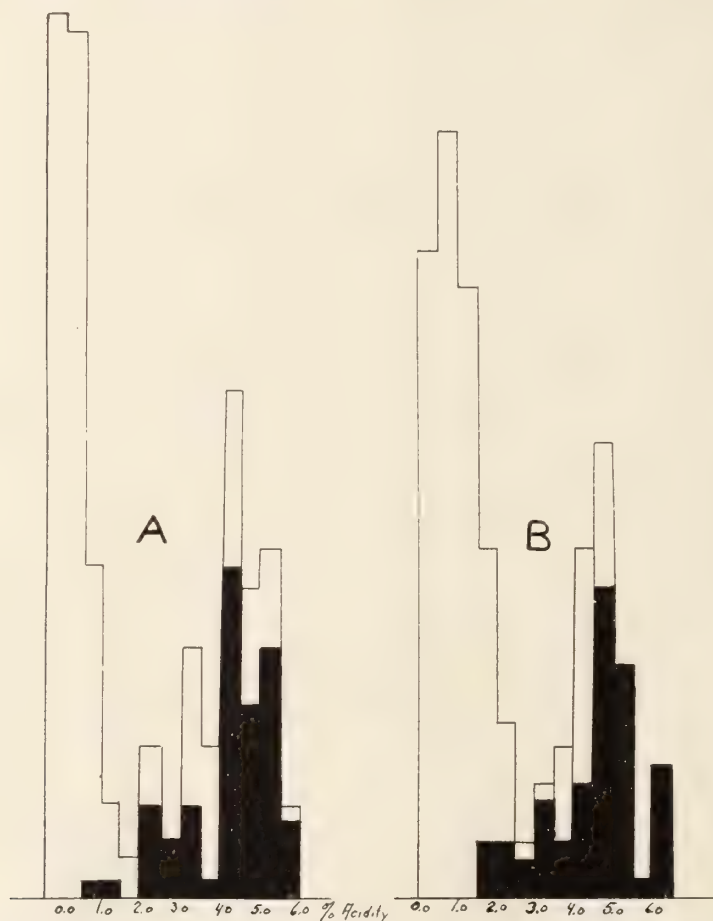


Fig. 3.—A. Frequency curve. Glucose in bottles. B. Frequency curve. Glucose in fermentation tubes. Shaded area = organisms producing gas in glycerol.

detected in 10 days. The advantage of obtaining data in less than 2 weeks instead of waiting 2 months need not be dwelt on.

Nitrate-reduction tests were made according to standard methods.

Indol determinations were made by Toby's method<sup>26</sup> after 8 days of incubation in Dunham's broth at 37 C.

<sup>26</sup> Jour. Med. Research, 1906, 15, p. 301.

Starch hydrolysis was followed by streaking starch plates and incubating them 1 week at 37 C. Iodin in KI solution was added to determine the disappearance of starch.

Spore tests were conducted on organisms by heating in glucose phosphate broth in Durham tubes at 80 C. for 15 minutes, using known colon bacillus as a control.

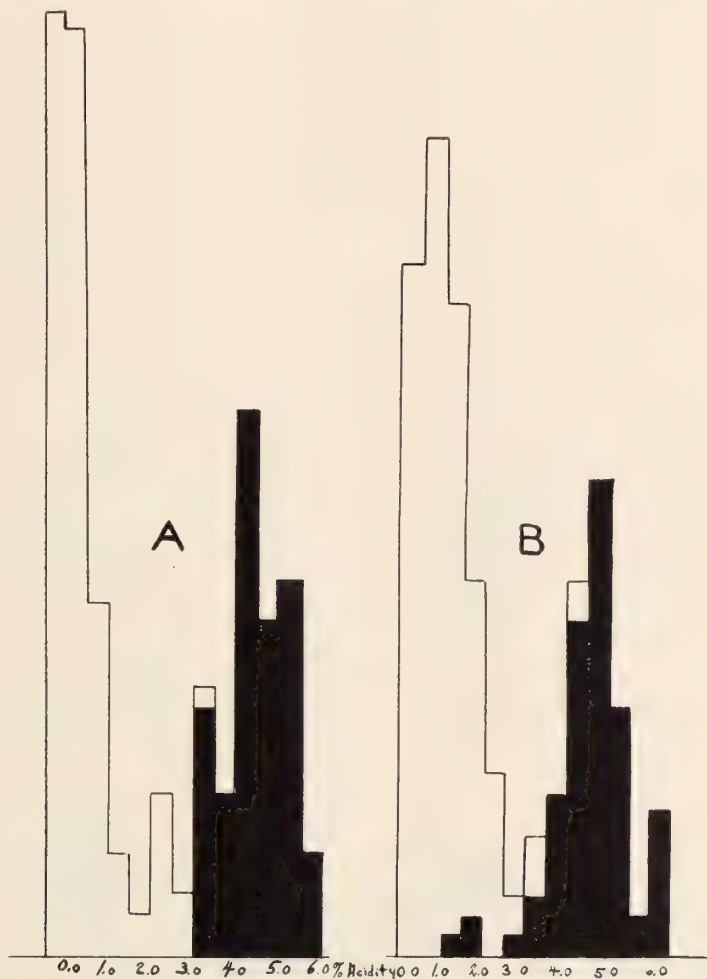


Fig. 4.—A. Glucose in bottles. Shaded area = organisms making sufficient acidity to show acid reaction of methyl red in bottle cultures in Medium II. B. Glucose in fermentation tubes. Shaded area = organisms making sufficient acidity to show acid reaction of methyl red in fermentation tubes in Medium II.

Frequency curves were plotted from the data obtained in the 2 sets of titrations of the glucose media (in test tubes and in bottles). The curves are reproduced in Figure 2. It can be seen that both have the

same general contour, and that both exhibit 2 modes; but, although it cannot be shown in curves of this size, the organisms in the low acid mode of 1 curve are not always in the low acid mode of the other

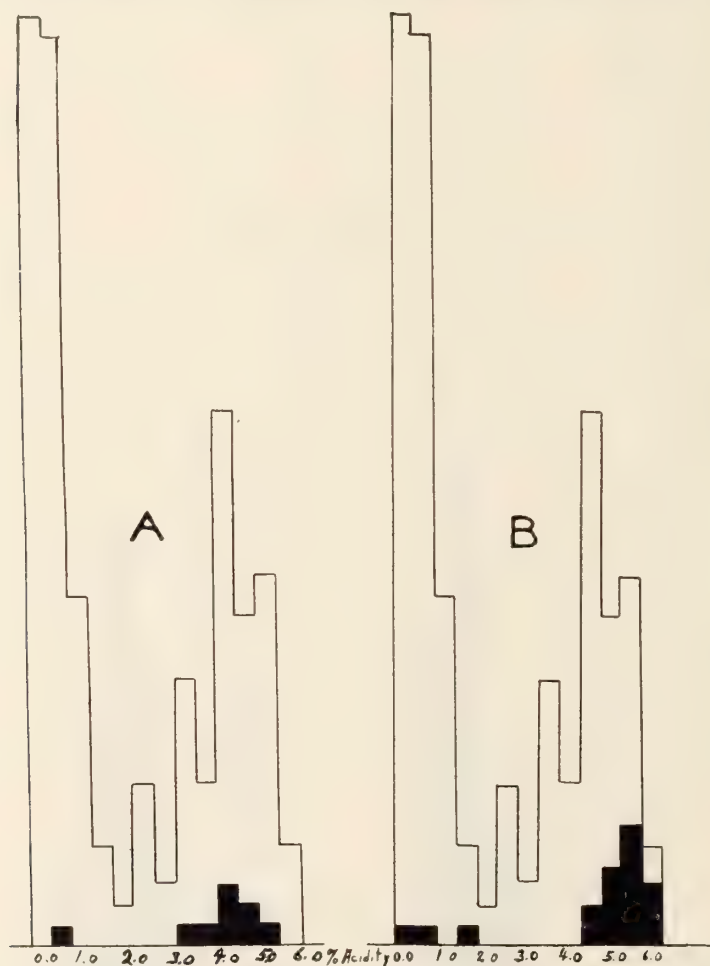


Fig. 5.—A. Glucose in bottles. Shaded area = organisms failing to ferment lactose with gas-production. B. Glucose in bottles. Shaded area = organisms failing to ferment saccharose with gas-production.

curve. An extended study of this point was made and the discrepancy ascribed to variability of acid-production. This subject will be taken up under the discussion of Stability and Variability of Characters. From all that we have been able to observe we are led to conclude

that the low ratio organisms remain quite constant, but that the high ratio strains produce small or large amounts of acid without any apparent law governing the phenomenon. This is shown in Figure 2 where the shaded area gives the location of the low ratio cultures in the frequency curves. It will be seen that they are confined to the high acid modes.

In seeking an explanation of this peculiarity of the high ratio organisms, contamination and differences in the degree of aeration may be excluded, for it was observed again and again under a variety of conditions. It will be shown later that the brand of peptone employed has a great deal to do with the variability. In all of the work described herein Eimer and Amend peptone was used, except where otherwise noted.

Figure 3 shows the correlation of the glycerol fermenters and the high acid mode. It is evident that the glycerol fermenters do not vary as much as do the nonglycerol fermenters.

Figure 4 shows the positions of the organisms which make sufficient acidity to give the acid reaction of methyl red when grown in the glucose-phosphate (II, p. 165) medium. This reaction should not be confused with the methyl red test of Clark and Lubs.\* It shows that when the organisms vary in the direction of high acid-production the hydrogen-ion concentration runs quite high, and the organisms begin to assume the characters of the low ratio type.

A few of the organisms failed to ferment both saccharose and lactose, although none failed to ferment one or the other of these sugars. The results are shown in Figure 5.

The Voges and Proskauer reaction is given by all methyl red negative organisms when the acid-production places them in the low acid mode, but when the acid-production runs high enough to place them in the high acid mode the Voges and Proskauer reaction may or may not be present. A possible reason for this will be apparent in the discussion of the mechanism of the variability. When employing the methyl red test in the prescribed manner of Clark and Lubs, that is, using 0.5% Witte's peptone, 0.5% glucose, and 0.5%  $K_2HPO_4$ , the Voges and Proskauer reaction given is always in the methyl red negative cultures. Occasionally there have been cultures in which the acid-

\* Clark and Lubs' methyl red test is made by inoculating a special medium whose composition has been previously stated, and incubating 5 days at 30 C. Two drops of methyl red solution are added to determine the reaction toward this indicator. Our use of methyl red, in addition to the bona fide Clark and Lubs method, was the addition of a few drops of it to our special medium (p. 5) to determine its reaction toward this indicator.



production was sufficient in our medium to give an acid reaction with methyl red and a positive Voges and Proskauer reaction.

The saccharose and lactose curves show 2 modes, but little is to be gained from a study of them.

*Summary.*—1. The frequency curve for the glucose fermentation, whether cultivation be carried out in fermentation tubes with little aeration or in bottles with much surface exposure, is approximately the same and shows 2 very distinct modes.

2. The methyl-red-positive organisms are to be found always in the high acid mode.

3. Certain of the methyl-red negatives produce sufficient acid in our medium (II, p. 4) to show the acid reaction of methyl red, and may or may not possess other supposedly correlated characters.

4. The organisms which ferment glycerin with gas-production seem to show a lesser tendency toward variability than do the non-fermenters.

5. There is little to be gained from a statistical study of the saccharose or lactose fermentations of this particular group of organisms.

6. Variation in acid-production occurs to a sufficient extent to cause some doubt as to whether the biometric method is of service in the study of this group of bacteria.

#### STABILITY AND VARIABILITY OF CHARACTERS

Variability is a term which is often inaccurately used. Strictly speaking, it means the temporary alteration of 1 or more characters, as contrasted with mutation, which implies permanent change.

Revis,<sup>27</sup> being of the opinion that the colon bacillus was originally a natural soil organism, believed that there should be a loss of certain functions when this organism is transferred to a new habitat, the intestine of man, in accordance with the usual behavior of parasites, and that, when returned to its original abode, it ought perhaps to regain some of its lost characters. Experiments to prove this with the colon bacillus failed, while those with *B. [lactis] aerogenes* seemed to give somewhat conflicting results. Continued cultivation of *B. [lactis] aerogenes* on synthetic media caused it to lose some of its characters apparently, such as the Voges and Proskauer reaction and the power of fermenting saccharose. This occurred in 2 instances, and the organisms became suspiciously similar to the colon bacillus, "the loss of the Voges and Proskauer reaction constituting a fundamental change."

<sup>27</sup> *Centralbl. f. Bakteriol.*, 1910, ii, Ref., 26, p. 161.

Rogers, Clark, and Evans<sup>7</sup> expressed the belief that the study of the Voges and Proskauer reaction would clear certain difficulties in the study of the colon group. This reaction, however, is not always constant and is not always correlated with other factors, as was expected by some workers. For instance, MacConkey<sup>28</sup> believed that a positive Voges and Proskauer reaction should be correlated with a definite gas ratio in the colon-aerogenes group, but frequently found it positive when the gas ratio was not characteristic. On the other hand, there was no tendency of the colon bacillus to alter its characters.

In 1909 he reported further on the variability of the Voges and Proskauer reaction and found in a few cases that it varies inversely with the indol-production, and to such an extent that he doubts the value of the reaction as a diagnostic measure. All of the Voges and Proskauer variants possessed the same fermentation reactions.

Levine has shown<sup>30</sup> that the Voges and Proskauer reaction is correlated with the methyl red negatives, while Johnson<sup>38</sup> found that not all of the presumably high ratio group were able to produce a positive Voges and Proskauer reaction. These observations are quite suggestive, in interpreting the work of others. Rogers, Clark, and Davis<sup>5</sup> and Rogers, Clark, and Evans<sup>3</sup> noted a constancy in the amount of gas-production and of gas ratio in low ratio organisms, while with regard to the high ratio group they repeatedly comment on its variability. They also found instances where an organism had apparently altered its gas ratio quite profoundly, but state that there may have been an error in lettering the cultures. This is undoubtedly the safer explanation, but in view of the results of others in this field, and of the results of our own investigation, the question of variability appears highly important.

Harden and his associates<sup>29</sup> have shown the Voges and Proskauer reaction to be due to a special decomposition of glucose by several organisms, chief among which are *B. cloacae* and *B. [lactis] aerogenes*, with the formation of large amounts of CO<sub>2</sub> and small quantities of hydrogen, together with a substance known as 2-3 butanediol, or butylene glycol, CH<sub>3</sub>-CHOH-CHOH-CH<sub>3</sub>. They further showed that in the presence of oxygen the organisms have the property of oxidizing the 2-3 butanediol to acetyl-methyl-carbinol, CH<sub>3</sub>CO-CHOH-CH<sub>3</sub> which is the substance that gives the Voges and Proskauer reaction. When the acetyl-methyl-carbinol is left in contact with air in the presence of strong alkali it undergoes oxidation to diacetyl, CH<sub>3</sub>-CO-CO-CH<sub>3</sub>, and if peptone is brought in contact with the diacetyl in the alkaline solution a pink compound is formed which usually possesses a greenish fluorescence. More recent work by the same investigators has tended to show that certain amino-acids are the agents in the peptone solution which have to do with the formation of the characteristic pink color of the Voges and Proskauer reaction.

Thompson<sup>30</sup> studied the chemical action of *B. cloacae* on glucose and mannitol, using *B. [lactis] aerogenes* and the colon bacillus for comparison. Some of his data like those of Harden are of particular interest in theorizing on the variability in this group. According to this investigator there is a marked resemblance between *B. cloacae* and *B. [lactis] aerogenes*.

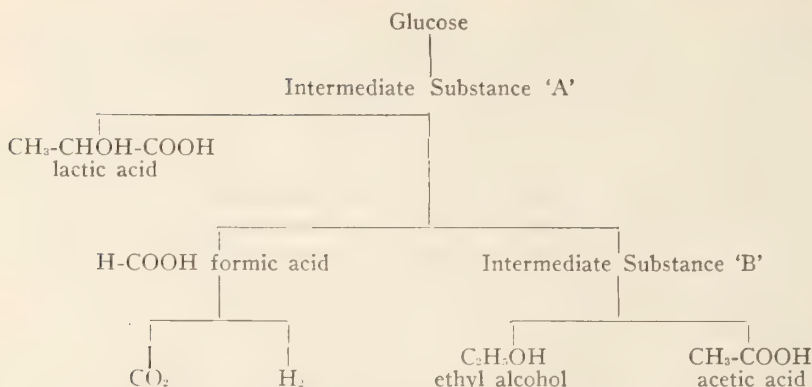
Grey's theory<sup>31</sup> of the decomposition of glucose by the colon bacillus is graphically represented as follows:

<sup>28</sup> Jour. Hyg., 1905, 5, p. 333.

<sup>29</sup> Jour. Physiol., 1911, p. 332. Proc. Roy. Soc., 1911, B 84, p. 492.

<sup>30</sup> Proc. Roy. Soc., 1911, B 84, p. 500.

<sup>31</sup> Proc. Roy. Soc., 1914, B 87, p. 472.



In the present problem the foregoing facts taken from the literature have led to the formulation of a theory which may serve to explain the inner metabolism of the 2 types of organisms, and incidentally to explain the mechanism of the variability. The theory is based on the following observations:

1. The low ratio organisms do not vary to any appreciable extent. They remain consistently Voges and Proskauer negative, and methyl red positive. They do not utilize all of the sugar present in the culture media, and the titrable acidity is always high, depending of course on the extent of the buffer action of the medium.

2. The high ratio organisms vary apparently without law, in several respects:

- (a) While they ordinarily produce little or no acid, there are times when they produce high acid, with a sufficiently high hydrogen-ion concentration to react acid to methyl red. When using Witte's peptone in the prescribed methyl red test the variability in this respect is small, and is confined chiefly to the yellow pigmented forms. But in certain American brands of peptone that were tried, the variability was so great that the assertion of Clark and Lubs that no other peptones than Witte's can be used is substantiated to a certain extent. This has led to a study of the influence of different peptones on the methyl red test and on variability, which will be presented later.

- (b) In addition to the variability in acid-production, there is variability in the amount of sugar used, and this variation is always parallel to the acid variation. Kendall<sup>4</sup> says, "Members of the cloacae group decompose glucose rapidly. Even at the end of 24 hours the sugar has disappeared from the culture medium, as is shown by actual sugar

determinations. The organisms therefore attack the protein and this explains the large amount of ammonia produced in glucose broth after the first day . . . . This is an important and permanent distinction between the colon and cloacae groups." In spite of Kendall's statement regarding the permanency of this point, it has been our experience that whenever the organisms of the cloacae group vary in the direction of high acid-production, the sugar utilization is never complete, as it so frequently is when the fermentation proceeds in what might be termed the normal manner for the cloacae group. There is a very definite variation in the amount of sugar used by the organism, depending on whether there is acid variation or not. When the acidity runs high very little sugar is used, and when the acidity remains low the sugar utilization is complete or very nearly so, depending on several factors, chief of which is the kind of peptone used and the length of time of incubation.

(c) When the variation from low to high acid occurs there is a tendency for the Voges and Proskauer reaction to disappear, and frequently when the acidity is high enough to show the acid reaction of methyl red in any sort of medium, either our glucose-phosphate broth, or Clark and Lubs' broth, the Voges and Proskauer reaction is extremely weak or totally absent. This might be ascribed to contamination, but frequent replating and selection of different colonies fail to give a constantly high acid-producing line in addition to the normal organisms. Furthermore, an organism will on one day behave in the usually accepted manner, that is, low acidity and complete sugar utilization, and on another it will vary in the direction of high acid-production, after which its behavior will be normal again.

The Voges and Proskauer reaction seems to be inseparably locked with the sugar utilization; hence, where little sugar is used the reaction is generally negative, or in doubt because of its faintness.

(d) When the acidity runs high and the sugar utilization is small, and when the Voges and Proskauer reaction is very weak or absent entirely, the amount of gas produced from glucose in the closed arm of the Durham fermentation tubes is not around 100%, as is usually the case, but is in the neighborhood of 50%, and as near as can be determined by this crude method the  $\text{CO}_2$  to  $\text{H}_2$  ratio is about 1:1.

Rogers and his associates have frequently suggested that there seem to be 2 parallel fermentations going on, one of which produces  $\text{CO}_2$  in great preponderance over  $\text{H}_2$ , while the other produces approx-



imately equal parts of H and CO<sub>2</sub>. They believe that the 2 reactions are mutually dependent, but the whole problem of variability in this group would be easily solved if it were shown that they are independent.

(c) The last important point in formulating the new theory is that whenever there is a variation toward high acid-production there is little growth in glucose media, and after a day or 2 the organisms begin to sediment out leaving a comparatively clear liquid which is quite comparable to the appearance of a glucose broth culture of the colon bacillus; whereas, *B. cloacae* when fermenting normally will give an extremely cloudy broth culture, which never clears.

If there are 2 reactions in the cloacae group, one of which is quite similar to the colon fermentation and the other to the 2-3 butanediol fermentation, as suggested above, then it is possible to explain the variability of this group with respect to amount and kind of gas-production, the titrable acidity, the hydrogen-ion concentration, the methyl red test which is dependent on it, the Voges and Proskauer reaction, the sugar utilization, and the amount of growth, providing the 2 reactions are considered independent to a certain degree.

Of course, contamination could explain the variability, but if a contaminating organism were present it would have to be one of the colon group, and a high acid former, and always the same organism. We appreciate the difficulty of excluding contamination, but it has never been our experience to meet the same contaminating organism in as many as 150 cultures. Again, the contamination would have to occur in the high ratio cultures only to be manifest. And lastly, contaminating organisms have been sought repeatedly and never found, except in 2 instances.

Of the 2 possible explanations the contamination idea seems to be the less likely, especially as MacConkey<sup>28</sup> has shown that *B. cloacae* grown in the presence of the colon bacillus will show the Voges and Proskauer reaction, and in many of our cases of variation the Voges and Proskauer reaction disappears entirely.

As far as is ascertainable from the data at hand there is no apparent rule which governs the variability, but it seems to be a matter of chance as to which fermentation will predominate. Assuming that the CO<sub>2</sub>-2-3 butanediol fermentation predominates and that the colon-like fermentation is subordinated, as is apparently the case in the 'normal' *B. cloacae* sugar decomposition, in accordance with the theory



that the 2 fermentations are independent, the following conditions will prevail in the glucose phosphate broth culture:

The fermentation will proceed with increasing rapidity until the sugar is all gone, because the end-products of the predominating type of fermentation are essentially nonacid in character and the 2-3 butanediol, according to Thompson, is a food for *B. cloacae*. The colon-like fermentation will proceed to a slight extent, and a very small amount of acid will be formed together with equal parts of  $\text{CO}_2$  and  $\text{H}_2$ . Following the loss of all the sugar there will occur more or less 'protein decomposition' depending on its availability, and thus a certain amount of ammonia-production will take place which would account for the alkalinity of some cultures.

2. There will be a very heavy growth in the tube, because of the absence of end-products of acid character to act as inhibitors, and because, as has been mentioned, the butanediol can be used further as a food.

3. A very large volume of gas will be formed, because the gaseous fermentation will not be stopped by anything other than the complete exhaustion of the sugar.

4. The gas ratio will be 'high', in the sense of Rogers and his associates, consisting of 2-4 parts  $\text{CO}_2$  and 1 part  $\text{H}_2$ .

5. The presence of the 2-3 butanediol will give rise, after bacterial oxidation, to acetyl-methyl-carbinol, which in turn will be responsible for the positive Voges and Proskauer reaction of this culture.

6. The acid-production being small, the subsequent alkali-production will permit of a very small hydrogen-ion concentration only, and if methyl red is added to the culture it will show an alkaline reaction.

A large volume of gas, high gas ratio, and low acidity seem to be inseparable.

On the other hand, let us consider what will be the situation resulting from the reverse of the previous conditions. In this case the colon-like fermentation will predominate and the  $\text{CO}_2$ -butanediol fermentation will be subordinated. For the time being, the cause of this reversal will not be considered. Step by step the following conditions will be found:

1. The fermentation will proceed until the acid produced from the colon-like fermentation will inhibit the growth, and practically no other change will take place in the culture. Only a small fraction of the sugar in the medium will be used. All reactions which may occur

will have to take place in the time elapsing between the time of inoculation and the time when the inhibiting acidity is reached.

2. If left in this condition for a few days most of the organisms will settle to the bottom of the tube, thus leaving a clear solution which will approach that of a culture of the colon bacillus under similar conditions.

3. Because of the rapidity with which the colon-like fermentation reaches the inhibiting acidity, or because the causal agent of the variation has slowed down the  $\text{CO}_2$ -butanediol fermentation, little  $\text{CO}_2$  will be formed by this method, and the volume of gas will therefore be small, approximating the volume expected from the colon bacillus under similar conditions.

4. The gas will approximate equal volumes of  $\text{H}$  and  $\text{CO}_2$ , with the latter probably slightly predominating, and the organisms will approach those of low ratio.

5. Because of the slowness or the lack of the  $\text{CO}_2$ -butanediol fermentation there will be few or none of the substances formed which go to make a positive Voges and Proskauer reaction and consequently the reaction will be negative.

6. The acid-production being high the cultures will give the acid reaction of methyl red.

A small volume of gas, low gas ratio, and high acidity seem to be inseparable.

A 3rd condition must be taken into account, namely, the failure of either fermentation to predominate. In such an event, it would be possible to have a certain amount of 2-3 butanediol formed before the inhibiting acidity is reached and would result in the paradoxical condition of an organism being simultaneously methyl red positive and Voges and Proskauer positive. The gas ratio would be lower than would be the case in the 1st of the foregoing assumed conditions, and higher than in the 2nd.

In the foregoing theoretical discussion reference is made to media whose composition was given on p. 165, and made of peptone other than Witte's. In Clark and Lubs' broth containing American peptones instead of the Witte all of the conditions do not hold, and it is suspected that this is due, at least in part, to the higher amino-acid content of the American brands as compared with Witte's peptone.

Table 4 shows the manner in which acidity varied and led to the study of the cause of variability. It is a collection of data taken from

cultures of the same strains at different times, except that those which are in the column headed 4/19/16 are from cultures which have been plated out for the purpose of once more detecting possible contamination.

TABLE 4  
A COLLECTION OF TITRATION FIGURES SHOWING VARIABILITY IN ACID-PRODUCTION

Number	Acidity Expressed as % Normal				
	3/9/16	4/1/16	4/4/16	4/12/16	4/19/16
6a contaminated.....	3.4	3.9	...	-0.5	...
6b .....	0.3	4.7	...	-0.3	...
7a .....	3.8	4.8	...	4.7	...
7b .....	3.2	4.8	..	4.5	...
9a .....	0.0	0.0	...	1.3	...
9b .....	0.0	0.0	..	3.9	...
11a P.....	3.4	4.5	...	3.2	...
11b P.....	3.8	5.0	...	2.6	...
12a .....	0.0	4.5	...	-0.4	...
12b .....	0.0	4.5	...	-0.3	...
13a .....	3.3	3.9	...	-0.4	...
13b .....	3.3	0.0	...	0.0	...
14a .....	4.2	4.0	...	5.3	...
14b .....	4.4	6.0	...	0.2	...
15a .....	4.1	6.7	...	-0.2	...
15b .....	4.2	7.0	...	0.2	...
16a .....	4.1	6.2	...	-0.2	...
16b .....	3.2	0.0	...	-0.1	...
22a .....	0.5	-0.3	...	4.1	...
22b .....	0.4	-0.7	...	3.4	...
23a .....	0.3	-0.3	...	2.9	...
23b .....	0.0	-0.4	...	2.4	...
24a .....	-0.2	0.1	...	2.4	...
24b .....	-0.2	0.2	...	0.0	...
29a .....	5.6	3.5	5.2	...	...
29b .....	4.2	3.9	4.1	...	...
31a .....	0.3	0.1	3.8	-0.2	...
31b .....	4.6	...	3.3	-0.4	...
32a .....	4.0	...	0.3	-0.3	...
32b .....	3.7	...	0.2	0.0	...
33a .....	2.3	...	0.2	-0.2	...
33b .....	4.2	...	0.0	-0.4	...
34a .....	4.0	...	0.0	-0.4	...
34b .....	2.6	...	0.0	-0.3	...
35a .....	0.0	...	3.6	-0.2	...
35b .....	0.0	...	3.8	-0.5	...
36a .....	0.1	...	4.3	-0.3	...
36b P.....	3.8	...	0.0	0.0	...
37a .....	4.3	...	0.5	-0.4	...
37b .....	4.4	...	2.3	-0.3	...
38a .....	3.6	...	3.1	-0.4	-0.2
38b .....	0.8	..	3.8	-0.3	3.6
39a .....	-0.2	...	3.9	-0.5	4.2
39b .....	0.0	...	3.8	-0.3	4.4
40a .....	0.0	...	3.8	-0.3	4.4
40b .....	0.0	...	3.7	0.0	4.2
41a .....	0.0	...	0.0	...	4.3
41b .....	-0.2	...	-0.3	...	4.3
42a .....	-0.4	...	0.0	...	-0.5
45a P.....	2.0	...	4.4	4.0	...
45c P.....	4.5	...	4.2	...	4.5
46a P.....	4.8	...	4.2	...	0.5
46b P.....	4.8	...	4.7	...	3.9
47a P.....	5.4	...	4.6	...	1.6
47b P.....	5.0	...	4.4	...	4.8
49a .....	-0.2	...	4.1	0.0	1.3
49b .....	-0.2	...	3.9	0.1	0.6
50a .....	0.0	...	4.3	0.0	0.0

P = pigment formers.

In Table 4 all of the organisms reported are presumably of the high ratio type, as was shown by Clark and Lubs' methyl red test. It will be noted that there is no apparent uniformity of variation, and parallel strains such as the 'a' and 'b' strains of a given number are not always subject to the same direction of variation at the same time. With the exception of 6a which was 1 of the 2 cultures that had been found contaminated, and the pigmented forms 11, 45, and 47, all have given reason to believe that they are high ratio organisms, but the pigmented forms behave in such a peculiar manner that it is impossible to state definitely whether or not they are high ratio also. The fact that they occasionally give the Voges and Proskauer reaction suggests the high ratio classification.

It may be seen why the biometric method in the classification of this group will be of little service. If data cannot be duplicated from time to time within the limits of very slight variability no system of classification can be based on these characters.

When an organism in this group varies it will be seen that the change is usually quite profound, and that there are few which might be called half variants. A culture is usually at one extreme or the other. This is further evidenced by the sharply defined modes of the frequency curves. Very few cultures are to be found in the intermodal space, while a few of the organisms which are found in the high acid mode would be expected to be in the low acid mode from their general method of metabolism, such as the presence of the Voges and Proskauer reaction.

If the 2 combinations of characters mentioned, namely, large volume of gas, high gas ratio and low acidity, on the one hand, and small volume of gas, low gas ratio, and high acidity, on the other, can be proved to be absolutely interlocked, as seems quite possible, then the correlations observed by Clark and Lubs which find expression in their methyl red test are easily explained. It can be seen that of necessity the methyl red positives gave a low gas ratio and vice versa; and further, that the correlation of methyl red negatives with Voges and Proskauer positives, as noted by Levine,<sup>10</sup> must necessarily follow.

Attempts were made to secure permanent mutants by fixing the variant in the high acid type of metabolism, in order to determine whether or not other characters varied with the glucose fermentation. All efforts of this nature were unsuccessful. It was thought that perhaps the colon bacillus might arise from *B. cloacae* or *B. [lactis]*

aerogenes when these are taken into the animal intestine, but cultivation of *B. cloacae* in the presence of bile at 37 C. during several transfers and then plating out did not result in the acquisition of any new characters, although there was evidence that the  $\text{CO}_2=2\text{-}3$  butanediol fermentation was somewhat restrained by this method of treatment, in comparison with the gas-production of the colon bacillus controls.

Cultivation in liquid Endo's medium or in broth containing Gentian violet, paranitrophenol, brilliant green or methyl red, was also unsuccessful. It may be of interest to note that the methyl red seemed to exert the greatest inhibition of all of the substances tried.

#### INFLUENCE OF DIFFERENT PEPTONES ON THE METHYL RED TEST

It has been stated by Clark and Lubs that no peptone other than Witte's would serve for the methyl red test, because in using other brands the results were inconsistent. If the results with other brands of peptone are inconsistent, the cause of this inconsistency must be some form of variability. The fact that all of our earlier studies were made with Eimer and Amend peptone, using meat extract and varying degrees of aeration, led to the suspicion that the variability might be a function of the medium as well as of the organisms. A set of experiments was planned which should elucidate the following points:

1. Is variability an inherent property of the cell or is it impressed by some outside forces?
2. What is the influence of one kind of peptone as compared with another?
3. What is the influence of meat extract?
4. What is the influence of aeration on the methyl red test?

To determine these points it seemed wisest to use a simple medium which would combine as many tests in a single series as possible. Such a medium was found in Clark and Lubs' broth, by substituting one peptone for another and by the addition of meat extract. Owing to a shortage of pure  $\text{K}_2\text{HPO}_4$ , the corresponding sodium salt was substituted, using the stoichiometrical equivalent, and in a series of tests comparing the 2 salts it was found that the methyl red tests gave identical results whether the potassium or sodium phosphate was employed.

Witte, Eimer and Amend, Armour, and Digestive Ferments Co. peptones were used.

Each brand of peptone was made up into Clark and Lubs broth and divided into 2 portions. To 1 part 4 gm. per liter of Liebig's meat extract were added. Both lots were then placed in test tubes and in the previously mentioned 4-ounce



glass bottles. The tubes and bottles were sterilized in the autoclave after a series of tests had been made to determine whether the medium containing Witte's peptone could be used with the same results when it had been autoclaved as when it had been sterilized intermittently. The results on 50 tests were identical. In each series of tests the following determinations were made: titrable acidity, methyl red reaction, presence of residual sugar, Voges and Proskauer reaction, and in a few instances the Sørensen titration.

Lastly a series of tests was conducted using meat extract alone in the place of peptone, and another using neither meat extract nor peptone, but Uschinsky's medium minus the glycerol.

As these experiments required about 1200 cultures the incubation time was cut from 5 to 3 days and the temperature raised from 30 to 37 C. Levine<sup>10</sup> recommends this change also. It was found that with respect to the methyl red test alone the results under the new temperature conditions were identical with those obtained by the prescribed method.\*

*Summary.*—The medium may in a small measure increase or decrease the variability of the organism, but the fundamental cause of variability is associated with the property of the high ratio cultures to bring about 2 parallel methods of sugar decomposition which are more or less independent.

When using Witte's peptone without meat extract the sugar utilization was never complete, and even when meat extract was added to the medium it was in many instances incomplete. The amount of sugar utilization was greater in the test tubes than by the bottle culture method. These results are quite in contrast to those obtained with Eimer and Amend peptone, in which in many instances the sugar completely disappeared without the aid of the meat extract. In the medium described on p. 165, sugar decomposition was even more often complete. This may have been due, however, to the fact that 1.0% peptone was employed instead of 0.5%.

There is, nevertheless, a marked difference in the action of different peptones, and it is to be inferred that this is referable to the amino-acid content, in amount or kind, of the peptones. This inference is strongly supported by Table 5, in which Witte's peptone is seen to contain the smallest amounts of amino-acids, as indicated by the Sørensen titration. It is with this peptone that the least variability has been observed.

In the modified Uschinsky medium without glycerol, but containing 0.5%  $K_2HPO_4$  and 0.5% glucose, there was little or no growth of the low ratio organisms, while the high ratio strains grew vigorously. Sugar utilization by the high ratio organisms was almost complete, and there is reason to believe that if sufficient time were allowed, all

\* Owing to their size the tables are not reproduced here. They may be found, however, in the unabridged thesis in the Yale Library, or duplicate copies thereof held by the authors.

TABLE 5  
COMPARISON OF SORESENSEN TITRATIONS OF CLARK AND LUBS' MEDIUM MADE WITH  
DIFFERENT PEPTONE

	Without Meat Extract		With Meat Extract		
Witte Peptone					
NH <sub>3</sub> .....	0.80	0.81	1.4	1.2	1.54
Amino-acids.....	0.55	0.60	0.6	0.5	0.61
Elmer and Amend Peptone					
NH <sub>3</sub> .....	0.75	0.85	1.3	1.3	
Amino-acids.....	0.86	0.89	1.10	1.22	
Dif Co. Peptone					
NH <sub>3</sub> .....	0.70	0.70	1.11	1.11	
Amino-acids.....	0.65	0.63	0.99	1.00	
Armour Peptone					
NH <sub>3</sub> .....	0.74	0.73	1.13*	1.20	
Amino-acids.....	0.99	0.91	1.21	1.30	

\* Average of 6 titrations on another lot of the same medium.

members of this group would entirely dispose of the sugar. The methyl red reaction is meaningless in this medium. On the other hand, it is of particular interest to note that the high ratio organisms are able to produce the necessary conditions for a positive Voges and Proskauer reaction, in spite of the fact that there is no added protein.

In the light of the foregoing biometric studies it may be said that biometry is of little or no value in the face of unrestricted variability. Unless one or the other of the 2 important characters of the fermenting organisms of this group, preferably the colon-like fermentation of the high ratio members, can be restrained, efforts to obtain dependable results will be unsuccessful.

*Conclusions as to the Influence of Medium and Methods of Cultivation.*—1. Variability must be reckoned with.

2. Witte's peptone seems to offer less opportunity for variation to the particular organisms under observation than do the other peptones which were employed, when used in the method prescribed by Clark and Lubs.

3. The advantage of Witte's peptone over the others is, however, comparatively small, in so far as these experiments show.

4. The final hydrogen-ion concentration reached by the several organisms in the different peptones is variable to a certain extent, and as far as can be seen from the data on hand, the method of cultivation, whether it be in tubes or in bottles, seems to influence the acid-production differently in different peptones, although the differences are slight.

5. A study of the nitrogen metabolism of the organisms, with a determination of the amino-acids necessary for their welfare, and a study of the influence of these amino-acids on the type of fermentation which predominates would be of great help in the investigation of variability of the cloacae-aerogenes group of bacteria.

6. Meat extract seems to contain some substances which are desirable as food for the organisms, because of the frequent increase of sugar utilization in the media to which it has been added.

7. A very strong Voges and Proskauer reaction is given in a phosphate-meat-extract-glucose medium which is apparently more reliable than the methyl red test, and on account of its lesser variability the Voges and Proskauer reaction is preferable.

8. The high ratio group organisms grow well in Uschinsky's medium containing glucose instead of glycerin, and give a Voges and Proskauer reaction. The low ratio organisms grow in it with difficulty.

#### QUALITATIVE CHARACTERIZATION OF ORGANISMS IN THE PRESENT COLLECTION

Since the biometric method has failed as an important aid in the classification of the organisms, recourse must be had to the older methods of identification based on qualitative differences. All that has been gained from the biometric studies here recorded may be summed up as follows:

There seem to be 2 subgroups of organisms which comprise the colon-aerogenes group. One of these is always methyl red positive and Voges and Proskauer negative, these characters being stable. The other group is variable, but its members usually are methyl red negative and Voges and Proskauer positive. Table 6 presents a system of classification with these 2 major groups as the basis.

The group number offers a convenient and easy method of recording qualitative differences, and of studying the numbers of organisms presenting similar characters. All organisms showing gas, although subsequent titration might show the culture to be alkaline, were considered as acid- and gas-formers, on account of the double reaction in

the sugar decomposition of the high ratio group. This idea was suggested by Rogers, Clark, and Evans.<sup>8</sup>

It is necessary to recall again that in this collection of cultures there are a few that come from sources which are questionable, that is, in which there may have been a possibility of pollution. These are designated as 'Discards.' Those which would be acceptable from the sanitary standpoint are designated as 'O. K.'

TABLE 6

DISTRIBUTION OF THE ORGANISMS OF THE PRESENT COLLECTION IN 13 OF THE GROUP NUMBERS OF THE CARD OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Group Number	Total Number Cultures	Number O. K.	Number Discards	Methyl Red Positives O. K.	Methyl Red Positives Discards	Probable Name of Group
(1) 121.1112011	9	9	0	0	..	?
(2) 221.1113031	22	12	10	6	8	?
(3) 221.1113032	124	93	31	0	0	B. cloacae Rogers Group D
(4) 221.1113532	13	10	3	0	0	Rogers Group C
(5) 221.1133032	2	1	1	0	0	?
(6) 221.1213031	2	0	2	..	2	?
(7) 221.1313032	1	1	0	0	0	Proteus ?
(8) 222.1112031	1	1	0	1	..	?
(9) 222.1113031	11	9	2	4	0	B. coli communior Rogers Group D
(10) 222.1113032	2	2	0	0	0	Rogers Group D
(11) 222.1133031	13	13	0	13	0	B. coli communior and acid lactic
(12) 222.1133033	1	1	0	1	..	?
(13) 222.1212031	1	1	0	1	..	?
Totals.....	202	153	49	26	10	

It can be seen from Table 6 that there is a very marked correlation between certain groups and the methyl red reaction, especially in the cloacae and in the colon-acidi lactici group.

Rogers, Clark, and Evans,<sup>8</sup> say, ". . . Species so established cannot be identified or separated from one another by the turn of a single characteristic. Natural groups of bacteria are bound together by the common possession of a certain combination of characteristics, no one of which is absolutely fixed."

According to this assumption, many of the group numbers which are frequently represented by very few organisms which differ from other group numbers in only 1 or 2 characters, but have in common many more characters, might be combined in several larger groups. (Of course, the observations of Rogers and his associates would necessitate keeping the organisms of presumably the same gas ratio together.)

On this basis the foregoing groups, numbered 1-13, consecutively, were regrouped into the following 6 'Types,'\* as shown in Table 7.

TABLE 7  
DISTRIBUTION OF THE 13 ORGANISMS OF THE PRESENT COLLECTION INTO 6 TYPES

All Sporeformers: Type I		121.1112011	No. of Representatives 9
High Ratio Organisms: Type II			
	Group		
	2	221.1113031	8
	3	221.1113032	124
	4	221.1113532	13
	5	221.1133032	2
	7*	221.1313032	1
	9	222.1113031	7
	10	222.1113032	2
Low Ratio Organisms: Type IV			
	Group		
	2	221.1113031	14
	6	221.1213031	2
	8	222.1112031	1
	9	222.1113031	4
	13	222.1212031	1
	11	222.1133031	13
	12	222.1133033	1

\* This has the group number of the proteus group but gives the Voges and Proskauer reaction, and is therefore included here.

To make the data in Table 7 more intelligible to those who are not accustomed to using the group number, the graphic and tabular method adopted by Rogers and his associates has been utilized. Because all of the cultures fermented glucose this sugar was not included among the tests.

Certain correlations may be noted: Of the 36 low ratio organisms, 26 produce indol, and 35 ferment glycerol with gas-production. In the whole collection exclusive of the known stock cultures there are 59 strains which can ferment glycerol with gas-production; they are distributed through the several types as shown in Table 9.

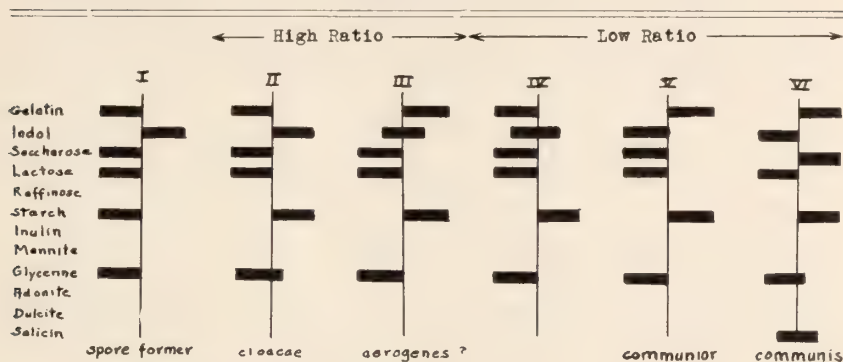
#### SUMMARY OF QUALITATIVE STUDIES

Thirteen group numbers have been represented by the collection of gas-formers found in nature. These have been regrouped into 6 types on the basis of the work of Rogers and his associates, in which acid-production from a sugar has been classed with acid and gas-production under the term 'fermentation.'

\* In working out these groups the known stock cultures were omitted.



TABLE 8  
GRAPHIC AND TABULAR METHOD OF DISTRIBUTION OF ORGANISMS



Type	Liquefaction			Decomposition of			
	Gelatin	Indol	Saccharose	Lactose	Starch	Glycerol	Salicin
I	9 100%	0 0%	9 100%	9 100%	9 100%	9 100%	
II	143 100%	2 1.3%	146 98.5%	147 99%	0 0%	148 100%	
III	0 0%	4 45%	9 100%	9 100%	0 0%	9 100%	
IV	16 100%	6 37.5%	16 100%	16 100%	0 0%	16 100%	
V	0 0%	6 100%	6 100%	6 100%	0 0%	6 100%	
VI	0 0%	14 100%	0 0%	14 100%	0 0%	13 93%	7 50%

TABLE 9  
DISTRIBUTION, ACCORDING TO TYPE OF STRAINS WHICH CAN FERMENT GLYCEROL WITH GAS-PRODUCTION

Type	Number of Strains	Cultures Positive, %
I.....	9	100
II.....	8	5.4
III.....	7	77.9
IV.....	16	100
V.....	6	100
VI.....	13	93
Total.....	59	

All of these 6 types may not be valid natural groups, especially those having few representatives, but there is reason to believe in the validity of Type I, the spore formers; Type II, the organisms of the cloacae-aerogenes group; and Types V and VI, which are the colon group proper.

Type II contains both pigment and nonpigment formers which give approximately the same sugar fermentations. The single representative of the group number 221.1313032 was included with this type, even though it does not utilize lactose, because it did not seem advisable to create another type for a single organism. (At this point it might be remarked that no recognizable members of the large proteus group have been encountered. Their habitat seems to be elsewhere than in the soil. From our experience and the publications of Kendall and his associates we infer that *B. proteus* (*vulgaris*) would be found with the low ratio organisms and would differ from them only in regard to the lactose fermentation.) The single organism mentioned above is a high ratio organism, gives a positive Voges and Proskauer reaction, and hence is in all probability not a member of the proteus group. Type II and Rogers, Clark, and Evans Group C are probably the same.

Type III should contain members of the *B. [lactis] aerogenes* group (nonliquefiers producing gas from glycerol). Seven representatives of Number 222.1113031 have been met with which are high ratio. These are probably the same as Rogers, Clark, and Evans Group 'D.' It is noteworthy that so few of the aerogenes group have been found in this research as compared with the large number of the cloacae group. In their paper, Rogers, Clark, and Evans report 40 cultures belonging to Group C and 90 to Group D. In our work we have found 148 and 9, respectively, in the groups which we believe correspond to their C and D groups. This suggests a different habitat for *B. cloacae* than for *B. aerogenes*. It may be, however, that an error in the determination of gelatin liquefaction accounts for the differences in the proportion of cultures in the 2 groups. There is no doubt that all of the liquefiers in this research are liquefiers, whereas incubation for 20 days at 20 C. leaves some doubt about the status of those which have not liquefied.

Type IV may be one of the types which would be classed with the colon bacillus by the ordinary methods of identification. At present nothing can be said of it to correlate it with some known group of organisms except that it has all of the reactions of the colon group, but yet liquefies gelatin.

Type V is probably the well known *B. [coli] communior* group.

Type VI probably contains members of the *B. [coli] communis* and *B. acidilactici* groups, although the cretaceous growth of certain of them leaves doubt as to their identity.

It is quite evident that *B. cloacae* is the predominating soil gas-former, in so far at least as the data here are concerned.

## GENERAL SUMMARY

About 1000 samples of soils, twigs, leaves, flowers, etc., have been examined for the presence of gas-formers. The organisms collected were arranged in 2 groups, one of which came from places where there is no reason to suspect fecal pollution of any sort, and the other from sources in which pollution is not an improbability. The organisms have been classified together and also separately.

An effort was made to study the cultures by the biometric method. Methods of cultivation and titration were given a critical study and attempts made to standardize them. Variability was encountered in the high ratio organisms to such an extent that the biometric method was of very little value in the classification work. This led to a study of the variability and metabolism of the high ratio organisms. A theory of the mechanism of the variability was formulated based on the apparent independence of 2 simultaneous methods of sugar decomposition. The correlations of the methyl red and the Voges and Proskauer reactions are readily explained by this theory, and the experimental results of the research appear to confirm the observation of Levine on this correlation.

Efforts to bring about mutation of the high ratio groups were unsuccessful.

The Voges and Proskauer reaction is apparently more dependable than the methyl red test.

The influence of specific peptones on the variability and the methyl red test has been studied, and while no very definite conclusion has been reached it was found that the peptone showing the lowest variability gave a medium of lowest amino-acid content.

It was shown that the substitution of meat extract alone for peptone in Clark and Lubs' medium gave somewhat variable methyl red tests, but the Voges and Proskauer reaction under such conditions was so strong as to be a deep red, instead of the customary pink obtained with peptones.

Uschinsky's medium minus glycerol, but with glucose added, furnished proper conditions for a Voges and Proskauer reaction, but is unsuited for the methyl red test. Low ratio organisms give very poor growth in this medium, while those in the high ratio group find it favorable for rapid multiplication.

## TUBERCULOSIS OF LYMPH NODES \*

FREQUENCY, ORIGIN, AND RELATION TO OTHER TUBERCULOUS LESIONS,  
ESPECIALLY PULMONARY TUBERCULOSIS

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The lymphatic system has important functions as shown so clearly in inflammation. In acute local inflammation local and later general swelling of lymph nodes is one of the first most pronounced and most important phenomena. The swelling consists in a proliferation of all the elements of the node, most markedly of the lymphocytes and leukocytes, and a production of immune bodies directed against the infection locally as well as generally. There are chemical affinities between microbes and their products and the cells of the lymph nodes, especially the leukocytes, and the struggle — the neutralization of the injurious effects — in the first instance takes place in the regional lymph nodes; later it extends to the nodes in general.

The same process occurs in chronic inflammations. This is shown most clearly in syphilis, the progress of which can be followed from place to place by study of the lymph nodes; first, there is a hyperplasia of the local lymph nodes, and when the infection has become generalized, there is a swelling of the lymph nodes generally. In leprosy, swelling of the lymph nodes is one of the first changes, and the nodes are the most important depots for the leprosy bacilli and become such so early, especially in the abdomen, that one might be led to assume that they are the first tissues to be attacked after the bacillus has passed through either the skin or mucous membranes, at the same time as the bacilli surely may persist in a latent condition in the nodes for years. At all events the leprosy process in the lymph nodes is a very chronic process and a very important localization.

In actinomycosis the conditions are different; it seems as if the actinomyces has little or no affinity for the lymphatic system. There is only little swelling of the lymph nodes in actinomycosis, and the fungus is hardly ever found in the nodes. It also appears that in the course of actinomycosis there is but a small number of immune bodies produced.

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In tuberculosis, on the other hand, the reaction in the lymph nodes is frequent and marked, just as in leprosy, the bacillus of which in many respects resembles the tubercle bacillus. Tuberculosis has a pronounced affinity for the lymph nodes, especially in childhood. In general, one may say that in children most of the tuberculous infections have their point of departure in tuberculosis of the lymph nodes; the tubercle bacillus is deposited here after it has passed through mucous membranes or the skin; here they proliferate enormously or remain latent but virulent for a long time, years and years, eventually escaping and infecting other organs. Most frequently the dissemination occurs by the lymph vessels, but also by the blood vessels and probably more often than now believed.

I now wish to discuss again tuberculosis of the lymph nodes and to use for that purpose the material which has accumulated in this institute during the last 12 years (1904-1915). This article is consequently a continuation of my larger article in 1905.<sup>1</sup> I wish to carry my investigations further and to discuss certain definite forms of tuberculosis of lymph nodes, more particularly the general form, its frequency in children and adults, its origin and importance as a cause of death. The common tuberculosis of the lymph nodes of the neck and of the bronchial lymph nodes is well known, but that tuberculosis of lymph nodes may be a general disease, that is, one which affects the whole lymph node system, is less well known; and yet it is not rare to find the lymph nodes tuberculous in all the most important groups, in the neck as well as in the chest and abdomen, and this occurs both in children and adults. This condition may be encountered incidentally in postmortem examinations; or, as is more commonly the case, it occurs in extensive, and as a rule eventually fatal, cases of tuberculosis, in which the process in the lymph nodes is the older and primary lesion, which as a rule causes tuberculosis in other organs and thus indirectly causes death.

Another point to be discussed especially is the connection between tuberculosis of the lymph node and tuberculosis in other organs, particularly with reference to pulmonary tuberculosis later in life, for the purpose of consideration from the anatomic point of view of the frequency of the so-called endogenous reinfection and tuberculous immunity, a problem that now is of special interest.

My observations are based on 2906 necropsies in 1904-1915, of which 2489 were of adults, that is, persons over 15, and 417 of children,

<sup>1</sup> Jour. Infect. Dis., 1905, 2, p. 142.



that is, persons under 15. Of these cases, 431, 14.8%, died of tuberculosis; including advanced tuberculosis in persons dead from other causes, we should have 501, that is, 17%. Of the adults, 351, 14%, died of tuberculosis; of the children, 80, or 19%. In 203 cases considerable tuberculosis of the lymph nodes was found, that is, in 7%, 127 cases, 5%, being in adults, and 76, 18.2%, in children.

Tuberculosis of lymph nodes falls into the following groups:

1. *Tuberculosis of the Bronchial Lymph Nodes.*—This constitutes a large group of primary tuberculosis of lymph nodes, including as it does, the bronchial lymph nodes, as well as the trachea and those in the hilus of the lungs. There were 57 cases of this form in all; in 8, however, the primary nature is uncertain; 38 occurred in adults and 19 in children, and all were fatal except 2. In most of the cases death resulted from a secondary miliary tuberculosis or tuberculous meningitis; this occurred in 16 adults and 14 children. In addition, there was often spondylitis, a mild pulmonary affection, and especially intestinal ulcer, which would seem to point to an additional infection by some other route.

A second large group of deaths are due to extensive secondary processes in the lungs, both in children, in whom it often is possible to demonstrate the direct point of entrance, and in adults; in this series there were 7 cases in children and 8 in adults. In many of these cases the clinical history gave previous 'glands in the neck,' of which there were no traces at necropsy. In these cases there was also tuberculosis in the bones and genital organs or disseminated miliary tubercles.

A third group includes secondary tuberculosis in bones, serous membranes, especially of the pleura or in the genitals (4 cases in adults) with small foci in the lungs, frequently at the same time.

There were 2 cases only of a latent tuberculosis of the bronchial lymph nodes.

A woman, aged 24, believed to have typhoid fever was found to have an extensive tuberculosis in the lymph nodes, in the thorax, and also pleuritis and salpingitis.

2. *Primary Extensive Tuberculosis in the Cervical Lymph Nodes.*—There were 10 cases, all in adults: 1 case in a girl, aged 15; 1 in a boy, aged 16; 5 in persons between 20 and 30, and the remaining 3 in persons aged, respectively 43, 45, and 50. Eight died of tuberculosis. In 2 the cervical tuberculosis was found incidentally. In 1 of the cases there was association with inveterate pulmonary tuberculosis. Of the

8 fatal cases, 2 died of miliary tuberculosis and 5 of secondary pulmonary tuberculosis, at times associated with intestinal and osseous tuberculosis. The 8th fatal case, which is of special interest, concerns a woman, aged 43, with lymphosarcoma in the abdomen, mediastinum, the glands of the neck, and metastases in different organisms. In addition there was tuberculosis in the lungs and in the lymph glands of the neck confirmed by inoculation of guinea-pigs.

Primary tuberculosis of the cervical nodes is comparatively rarely demonstrated, and it should be borne in mind that tuberculosis here frequently passes away without leaving any signs. This may also be true in extensive and widespread tuberculosis in other groups of lymph nodes in which it is learned that the disease began with swelling of the cervical nodes; anatomically, there may be no trace.

3. *Old Primary Extensive Tuberculosis in Abdominal Lymph Nodes.*—Here we have only 9 cases, in 7 adults and 2 children, only 3 dying of tuberculosis, 2 adults and 1 child, the others being found incidentally. In the cases in which the patients died from tuberculosis there were localizations in the bones, the urogenital tract, and the adrenals. As indicated, small old tuberculous changes frequently found in these groups of glands are not included.

Of special interest is the case of a woman, aged 78, who had had chronic obstruction for many years, and 6 years before death digestive difficulties and abdominal pains, and during the last year of life abdominal pain again and attacks of fever; finally a pulmonary process and pleuritis developed. Caseous and calcified lymph nodes were found in large numbers in the abdomen, and a recent tuberculosis of the lungs and pleura.

4. *Extensive Old Chronic Lymph-Node Tuberculosis in the Neck and Chest.*—This group contains 40 cases, of which 3 are doubtful; 20 occurred in adults and 20 in children. In 23, 9 adults and 14 children, the process caused death, and in 17, 11 adults and 6 children, the process was found incidentally. Of the 23 who died from tuberculosis, 9 (2 adults and 7 children) died of a secondary lung tuberculosis, 7 (2 adults and 5 children) of miliary tuberculosis and tuberculous meningitis, 2 (1 adult and 1 child) of tuberculosis of the serous membranes, 1 child of spondylitis, 1 adult of intestinal tuberculosis, 2 adults of urogenital tuberculosis, and 1 of secondary amyloid degeneration. Of the 17 cases found incidentally, there were several, both in adults and in children, in which the tuberculosis of the thoracic nodes appeared

to be an old affection, while the cervical tuberculosis appeared comparatively recent, perhaps an expression of infection through different paths and at different times.

It may be of interest to mention a few individual observations:

A man, aged 38, who had 'glands in the neck' in youth, developed tuberculosis in the lungs and larynx, and a retropharyngeal tuberculous abscess. There were large caseous lymph nodes in the neck and chest.

A man, aged 19, had suppurating glands as a child; he died of pulmonary and intestinal tuberculosis. There were large caseous glands in the neck and chest.

A woman, aged 35, died of miliary tuberculosis after having had 'glands in the neck' for 16 years. There were old tuberculous nodes in the hilus of the lungs.

A woman, aged 48, died of amyloid degeneration and extensive thrombosis. In the neck were large continuous masses of old tuberculous lymph nodes, similar masses also in the chest and an old pulmonary tuberculosis, descending tuberculosis of cervical nodes.

5. *Primary Contemporaneous Old Tuberculosis in Cervical and Abdominal Lymph Nodes.*—Of the 4 cases in this group, 1 was fatal (adult), while the other 3 were discovered incidentally (2 adults and 1 child).

A boy, aged 13, died rather suddenly of diphtheria. He had been treated for 'glands in the neck', and tuberculous ulcers were found on the leg, and near the anus, and also tuberculosis in the lymph nodes of the neck, the groin, and the pelvis.

A boy, aged 16, died of poisoning with resorcin. He had tuberculosis in the cervical and mesenteric lymph nodes.

A woman, aged 30, had ulcers on the legs for 10 years; also enlarged glands in the neck, anemia, albuminuria, and finally lung symptoms and enteritis. There were found tuberculous ulcers in the intestine, a small tuberculous focus in 1 lung and extensive amyloid change; numerous firm nodes in the neck from the jaw to the clavicle, the largest the size of walnuts, and a similar old caseous tuberculosis in the lymph nodes in the abdomen, retroperitoneally, in the hilus of the liver, in the hilus of the spleen, about the stomach, and in the mesentery. Presumably an infection of the lymph nodes of the neck and abdomen had taken place years back.

6. *Extensive Inveterate Tuberculosis of the Thoracic and Abdominal Lymph Nodes.*—This group includes 22 cases, 17 in adults, of which 14 were fatal, and 5 in children, aged 12-14 years, were all fatal. The cause of death was miliary tuberculosis, 1 adult and 1 child; primary (?) intestinal tuberculosis, 2 adults and 2 children; urogenital tuberculosis (3); tuberculosis in the adrenals with Addison's disease (3); tuberculosis of serous membranes (1); secondary (?) pulmonary tuberculosis (3); tuberculosis in bone, pericarditic pseudocirrhosis in a boy of 13; and a peculiar symptom complex with fever and anemia.

The last case occurred in a woman, aged 30 or 40, who suffered for half a year with fever and increasing anemia. There was a small calcified focus in 1 lung. Along the arch of the aorta there were several caseous lymph nodes of the size of chestnuts. Similar caseous nodes were in the hilus of the spleen and vicinity. There was a large spleen with numerous pea-sized tuberculous foci; and there were also numerous tubercles in the liver.

It seems most reasonable to regard the localizations in these 2 large groups of lymph nodes as closely connected and as a rule not due to independent infection.

*7. General Lymph-Node Tuberculosis.*—This is the most important group, and includes 61 cases, those of 32 adults, and 29 children.

Of the cases in children, 20 occurred during the first 3 years of life, and in almost all there was tuberculosis in the immediate relatives, most often in the parents. There were cases in older children, 4 in children aged 14. At the same time extensive tuberculous processes were found in internal organs, so that it usually was impossible to trace the connection, but the process in the lymph nodes was clearly the most marked and oldest. In all these cases, with 1 exception (Case 25) tuberculosis was the cause of death.

1. Child, aged 1½, died of intestinal tuberculosis and tuberculosis of the hip joint. There was a marked general lymph-node tuberculosis, apparently oldest and most advanced in the thoracic nodes, with extension to the lung and caseous pneumonia.

Presumably this was a case of primary tuberculosis in the thoracic lymph nodes, or possibly an intestinal tuberculosis, or a simultaneous infection in the chest and abdomen.

2. Girl, aged 14, whose mother died of consumption, had 'glands' for many years, and died of amyloid. There was an extensive old and continuous lymph node tuberculosis on both sides of the neck with adhesion to the lungs, in the posterior and anterior mediastinum, hilus of the lungs, and along the bronchi; also old tuberculous intestinal ulcers, with tuberculosis of the corresponding lymph nodes. There were, furthermore, small indurated tuberculous foci in the apices of the lungs, tuberculous endometritis and salpingitis, and amyloid degeneration.

This no doubt was a primary tuberculosis in the cervical lymph nodes, with extension (?) to the glands in the chest, either by continuity or by way of the circulation.

3. Child, aged 4, had chronic trouble in the eyes and nose, and disease of the intestines for about 2½ years. An old intestinal tuberculosis, especially in the cecum with tuberculous peritonitis, was found and also a general lymph node tuberculosis of earlier origin. In the posterior mediastinum, the apex of 1 lung was adherent to a lymph node and a small tuberculous focus and cavity had formed. There was also miliary tuberculosis, tuberculous menin-



gitis, large tuberculous masses in the brain, and tuberculosis of the endocardium and the left ventricle.

In this case the intestinal tuberculosis was probably the oldest process and may have given rise to the tuberculosis in the lymph nodes.

4. A child, aged 10 months, had large caseous masses in the anterior mediastinum, along the trachea, in the hilus of the lungs and also along the bronchi, with rupture of a bronchus, and focus in the lung; some small tuberculous nodes in the neck and the retroperitoneal tissue; and large masses in the mesentery.

This was probably a case of primary tuberculosis in the thoracic lymph nodes without direct connection with other groups of nodes.

5. Child, aged 5, who had had persistent bronchitis since 3; had cavernous pulmonary tuberculosis, tuberculosis of the intestines and of the largest lymph-node groups in the neck, the chest, and abdomen. Nothing can be said about the connection.

6. Artificially nourished orphan, aged 1 year, had cavities in the lungs, and large tuberculous masses in the lymph nodes of the chest, neck, and abdomen. Nothing definite can be said about the course of events.

7. Child, aged  $2\frac{3}{4}$  years, presented a marked general tuberculosis with perforation of a bronchus and a small pulmonary focus. Apparently there was a primary lymph-node tuberculosis of about the same extent everywhere.

8. Child, aged 1 year, whose mother died of tuberculosis, had diarrhea and then lung symptoms. There were a marked general lymph-node tuberculosis, with perforation in 1 lung, tuberculous ulcers in stomach and intestine, and scattered miliary tubercles.

The lymph-node tuberculosis was probably primary, but whether it developed from several points or spread from 1 group could not be determined.

9. Child, aged 10 months, had meningitis and miliary tuberculosis. There was general tuberculosis of the lymph nodes of the neck (comparatively recent), along the trachea, in the hilus of the lungs, and along the bronchi; also in a retroperitoneal gland, but not in the mesentery.

In this case the tuberculosis in the thoracic nodes undoubtedly was the oldest and spread to the other groups.

10. Child, aged  $1\frac{1}{2}$  years, died of miliary tuberculosis and tuberculous meningitis. There was a general lymph-node tuberculosis, most marked in the chest and neck and undoubtedly primary in 1 or the other.

11. Child, aged 2 years, had tuberculous ulcers in the intestines and a large tuberculous growth in the cerebellum; furthermore, tuberculosis in the lymph nodes of the neck and chest, larger in the thorax, and also in the mesentery.

As the process was oldest and most marked in the chest, it is possible that the infection in the neck was secondary thereto.

12. Child, aged 7 months, had a marked tuberculosis of the lymph nodes, most pronounced in the mesentery and behind the peritoneum; next on both sides of the neck and in the axillae, and in the chest. There was also a beginning pericarditis and pleuritis and some scattered tubercles in internal organs.

The process was most marked in the abdomen, and possibly primary here, the dissemination being either by the lymph vessels or possibly by the blood vessels.

13. Child, aged  $1\frac{1}{2}$  years, had chronic intestinal symptoms for some time. There was marked old tuberculosis of lymph nodes, most advanced in the



neck, but present also along the trachea and bronchi, and in the abdomen. There were also tuberculous intestinal ulcers, meningitis, and a tuberculous focus in 1 lung.

In this case the process was oldest in the neck and chest and in the intestines; but the course of events was not clear.

14. Child, aged 1½ years, whose mother was tuberculous, died of pulmonary and intestinal tuberculosis and had an extensive process in the glands of the neck, chest, and abdomen.

15. A child, aged 8 months, had general lymph-node tuberculosis, there being large caseous nodes in the neck, largest in the upper part, also in the chest and abdomen, and a large tuberculous focus in 1 lung in direct connection with tuberculous bronchial lymph nodes. There was no intestinal tuberculosis.

The process was most marked and probably earliest in the neck and chest; the abdominal process was probably secondary.

16. A girl, aged 9 years, whose mother was consumptive, died of miliary tuberculosis and tuberculous meningitis. There were some fibrous tuberculous nodes in the neck, in the hilus of 1 lung, and in the mesentery, also tuberculous ulcers of the intestine.

Here was a disseminated, partly localized tuberculosis in different groups of lymph nodes. The intestinal ulcer could hardly be a point of origin for all these localizations, which probably arose from distinct infections at different times.

17. Child, aged 8 months, both of whose parents were tuberculous had caseous lymph nodes in the neck, numerous large caseous nodes in the chest, in the mesentery, and also retroperitoneally; secondary pulmonary tuberculosis and recent tuberculosis in the intestine.

In this case the lymph-node process was surely primary, and the lymph nodes in the chest appear to have been most affected.

18. Girl, aged 8 months, whose mother was consumptive, died of miliary tuberculosis. There were large caseous glands in the neck, chest and abdomen, a pronounced primary lymph-node affection, but not continuous.

19. Girl, aged 14 years, had 'glands' when young and tuberculous peritonitis 2 years before death. There were cavernous pulmonary tuberculosis and caseous pneumonia, intestinal and peritoneal tuberculosis, and caseous lymph nodes, especially in the neck, but also in the chest and abdomen.

On account of the extensive changes, the connection between the different processes could not be determined, but the history indicated an early lymph-node tuberculosis.

20. Boy, aged 5 years, had 'glands' for many years with open scars and sores, finally, enlargement of the abdomen, a general lymph-node tuberculosis with caseation, the masses in the abdomen being especially large, also a severe pulmonary and intestinal tuberculosis.

While no definite statement can be made as to the connection, the history pointed to an early tuberculosis in the lymph nodes.

21. Child, aged 7½ months, whose mother died of galloping consumption when the child was 4 years old, had an extensive tuberculosis in the thoracic lymph nodes, with rupture into a bronchus and caseous pneumonia. In addition, there were smaller tuberculous nodes in the abdomen and in the neck, and miliary tubercles in different organs.

It would seem reasonable to assume that in this case the tuberculosis of the thoracic nodes was the oldest and gave rise to the processes elsewhere.

22. Boy, aged 3 years, had caseous lymph nodes on both sides of the neck, extending from above into the thorax, being connected directly with similar caseous masses along the trachea, in the hilus of the lungs and along the bronchi. There were perforation of a bronchial node with a local pulmonary focus, caseous lymph nodes in the mesentery and behind the peritoneum, scattered miliary tubercles, and a large conglomerate mass in the cerebellum.

There may have been a direct connection between the process in the neck and chest, but separate infection at different times was also possible.

23. Girl, aged 6 years, had general lymph-node tuberculosis, caseous nodes on both sides of the neck, along the trachea, in the hilum of the lung and along the bronchi, perforation of the bronchus, pulmonary and intestinal tuberculosis, and caseous masses in the mesentery.

Nothing definite can be said about the connection. The process in the neck seemed to be of long standing, and the mesenteric nodes may have become infected from the intestine.

24. Boy, aged 2 years, had extensive lymph-node tuberculosis in the usual places in the chest, with caseation and perforation of the right bronchus, and secondary lung tuberculosis; caseous lymph nodes in the lower parts of the neck and in the abdomen; and tuberculous meningitis. It appeared as if the lymph nodes in the chest were involved to the greatest extent, and possibly primarily.

25. Child, aged 15 months, whose mother died of tuberculosis, had hydrocephalus, extensive lymph-node tuberculosis, especially in the chest and on both sides of the neck, smaller caseous nodes in the mesentery and inguinal region, miliary tubercles in the spleen and liver, and tuberculous ulcer in the ileum. The oldest process seemed to be that in the neck and chest, but there was no direct communication between these groups.

26. Girl, aged 14 years, whose father was probably tuberculous, died of miliary tuberculosis, having had pleuritis 2 years back and steadily growing glands in the neck for the last 8 months of life. There were large masses of caseous lymph nodes on both sides of the neck, smaller ones along the trachea, in the hilus, and along the bronchi, with rupture into the lungs, and scattered pulmonary foci. There were caseous lymph nodes in the minor omentum and behind the peritoneum.

The process in the neck was most marked and of long standing. Apparently there was a direct connection between the process in the neck and in the chest.

27. Child, aged 1½ years, died of tuberculous meningitis. There was an extensive tuberculosis in the thoracic nodes, also less marked tuberculosis in the glands of the neck and mesentery.

In this case the thoracic nodes were undoubtedly affected primarily; probably the other nodes were infected by way of the blood.

28. Boy, aged 14 years, 4 of whose sisters and brothers died of tuberculosis, died of pulmonary tuberculosis. There were an extensive cavernous tuberculosis in the lungs, with gelatinous pneumonia, perforation of the bronchus by tuberculous node and fatal hemoptysis, intestinal tuberculosis, extensive old tuberculosis in the trachea, in the hilus and along the bronchi, and a few caseous glands in the neck and abdomen, both in the mesentery, as well as along the aorta and the iliac arteries.

The changes are so extensive that it is impossible to determine whether the thoracic lymph nodes or lungs were primarily infected.

29. Boy, aged 2 years, whose mother was tuberculous, died during hemoptysis. There were extensive cavernous tuberculosis with gangrene and empyema.

scattered miliary tubercles, and marked caseation in the lymph nodes in the neck, axillae, hilus of lungs, and abdomen.

The process in the lungs was so extensive that the primary point of origin could not be determined definitely, but probably the process in the lymph nodes was the primary one.

These cases show that the lymph nodes in the neck, in the thorax, and in the abdomen were markedly and extensively affected. Occasionally, there was also a similar process in the axillary and inguinal nodes. Frequently there was a descending process in the neck, most marked above, and reaching down into the supraclavicular spaces; next in the thorax along the trachea, least marked above, down to the bifurcation and pulmonary hilus where the masses usually were largest, and then along the bronchi. Apparently, there was a continuous series of infected lymph nodes, but it must be noted that the extent decreases downward in the neck and upward along the trachea.

In the abdomen there was generally a more isolated tuberculosis, either in the mesenteric nodes only or in the retroperitoneal nodes, sometimes in high degree and in the upper part of the abdomen, along the aorta, the celiac axis, or in the hilus of the liver and spleen.

Consequently, in general, it does not appear that the coarser anatomic conditions point to a continued and continuous spread along the lymph vessels from one point and from one place to another. In the case in which the process in the lymph nodes was found incidentally, it was marked in the thorax and the neck, but was only in the early stages in the mesenteric nodes. For the present no consideration is given to the question whether there are directly communicating channels between the different groups of lymph nodes.

On the other hand, the impression is frequently gained that a repeated infection by different paths and of different places may have occurred, especially when the glandular process clearly appears to be of different ages, old, perhaps obsolete, in one long locality and recent in another which has no direct connection with the first. That this would frequently be the case is indicated also by the presence close at hand of a rich source of infection, such as a consumptive parent, so that the child is exposed to infection at different times by inhalation as well as by swallowing.

There remain a number of observations of extensive tuberculosis of the lymph nodes in all the regions, perhaps more marked in some particular group and, if so, usually in the chest or neck. Frequently the information is supplied that the disease began as an infection of

the glands of the neck which has been observed through months and even years. In these cases one may assume either a continuous spread by way of the lymph vessels from group to group, which no doubt occasionally does take place, or a dissemination by way of the blood. After the bacilli have accumulated in considerable numbers in one place in the bodies of children, they soon spread very rapidly, at first mostly by the lymph vessels or directly into the blood vessels, including large veins. That this may be the course of events is shown by the general experience that hematogenic dissemination in internal organs regularly takes place, tuberculous meningitis or miliary tuberculosis being the end. As the lymph nodes are good culture grounds for the bacilli they soon become infected either directly from the blood stream or indirectly through the lymph from different organs infected by way of the blood. Such, no doubt, is frequently the course in children, especially young children.

In 1905 I<sup>1</sup> published a series of observations supporting the view now advanced; by microscopic examination and inoculation of guinea-pigs, I showed that a latent tuberculosis is often present in several groups of lymph nodes at the same time, especially in the bronchial and cervical groups, or in the bronchial and mesenteric, or in all 3 groups. Even old healed forms and fibrous and hyaline tubercles were found in all 3 groups in 2 children, aged 10 and 14, respectively. Latent bacilli were also frequently demonstrated in different groups of lymph nodes apparently normal, though a little enlarged; for example, simultaneously in the nodes of the neck and mesentery, less frequently in the nodes of the neck and chest, and 5 instances in all 3 groups.

Similar observations are recorded by de Besche,<sup>2</sup> who made systematic inoculations of lymph nodes, especially from the neck and mesentery in children. He found a simultaneous infection in no less than 33 cases in 46, that is, 70.4%, but as a rule, as distinct infections. He found a simultaneous general infection in 8 of 14 cases of latent tuberculosis and in 7 of 10 cases, in which there were latent tubercle bacilli.

Ungermann<sup>3</sup> inoculated lymph nodes systematically from the bodies of 171 children and obtained tuberculosis in 34, that is, 22.8%, 29 having died of tuberculosis, 10 had a latent infection, and in 4 there were latent tubercle bacilli without any anatomic changes. When a group of lymph nodes was found to be tuberculous, there was generally infec-

<sup>2</sup> Bakteriologiske studier over barnetuberkulose, Christiania, 1912.

<sup>3</sup> Tuberk.-Arb. a. d. k. Gsndhtsamte., 1912, No. 12, p. 213.



tion of the other 2 groups. The bronchial nodes were most extensively infected and apparently the infection there was of the longest standing. He found a general lymph-node infection in 30 cases, 76-77%. He assumes that as a rule the nodes in the chest are infected first or possibly the mesenteric nodes and that dissemination by the blood carries the infection to the other groups; further, that the nodes in the lower part of the neck may be infected by way of the lymph vessels from the nodes in the chest, but only in slight degree. Hematogenic infection is the rule, and most often from the thoracic nodes, which are infected again by way of the lymph vessels from the lungs following inhalation of tubercle bacilli.

This is in harmony with my observations and probably applies to the cases of general lymph-node tuberculosis in childhood. A rapid extension occurs along the lymph vessels after 1 or more infections of different groups and then a hematogenic dissemination through the whole system and all groups of lymph nodes, which enlarge and become tuberculous, so that before long it is impossible to trace the route or mode of infection.

As to the cause of death in general lymph-node tuberculosis of children, it appears on the basis of this material that pulmonary tuberculosis is the cause of death in about one half of all cases, as so frequently is the case also in isolated tuberculosis of the bronchial nodes. Not rarely tuberculosis of the lungs assumes a phthisical form with cavities, as is often seen, particularly in older children, but not rarely in the first years of childhood and even in infancy. The lungs are attacked much less frequently through the coalescence of lymph nodes and the apices of the lungs, as may happen in the regions above the clavicles and in the upper part of the mediastinum. In certain cases it is impossible to decide whether the process of the lungs is the point of origin of the tuberculosis of the thorax and elsewhere.

In about one fourth of all of the cases tuberculosis in the intestinal tract is the principal lesion and cause of death, and presumably the primary infection developed as a tuberculosis in the abdominal lymph node with extension to the other groups of nodes.

Naturally, the cause of death often is a miliary tuberculosis or a tuberculous meningitis, which is the case in about one fourth of all the cases, as is so often the case in tuberculosis of the individual groups of lymph nodes.

Greater interest is aroused by general lymph-node tuberculosis in adults. In the years 1904 to 1915 I observed 32 cases, of which 30



were directly fatal and 2 were discovered incidentally. As there were 3489 necropsies in adults in this period, this is 1.3%. There were altogether 127 cases of extensive tuberculosis of lymph nodes, that is to say, 5% of all persons examined after death.

1. Man, aged 32, had pleuritis at the age of 14, and suffered from digestive disturbances for 4 years. There was an old and severe intestinal tuberculosis with strictures and extensively caseous lymph nodes in the mesentery and behind the peritoneum. There were caseous lymph nodes as large as walnuts in the supra-maxillary fossa along the neck muscles, the trachea, and in the hilus of the lungs. There was, finally, a pulmonary tuberculosis of comparatively recent date. It would seem likely that this was a case of general lymph-node tuberculosis starting in the abdomen.

2. A man, aged 29, died of a chronic tuberculosis of the lungs, with cavities. There were also tuberculosis in the larynx and intestine and an old general lymph-node tuberculosis in the chest, neck, and abdomen, but nothing definite could be determined as to the connection and extension.

3. A woman, aged 22, had, as a child, 'glands' and died after symptoms of lung tuberculosis for about 1 year. In addition to a cavernous process in the lung there was tuberculosis of the intestine and a general lymph-node process in the neck, some of the nodes being calcified, in the clavicular fossae, in the axillae, along the trachea and bronchi, and in the mesentery and retroperitoneal tissues, but the process was too far advanced to permit any definite inference as to the mode of extension, though the process in the neck was very old and a spread downward would seem possible.

4. Woman, aged 21, died of puerperal infection. Tuberculous lymph nodes the size of walnuts were present on both sides of the neck, in the left axilla, along the bronchi, but smaller there, also in the mesentery and behind the peritoneum, where they were completely caseous. This then is a case of general lymph-node tuberculosis most marked in the neck and abdomen, but anatomically not connected. Did the spread take place by way of the blood from the neck or abdomen or did infection occur by different routes at different times?

5. Woman, aged 30, was of a tuberculous family. In addition to pulmonary and intestinal tuberculosis and tuberculous peritonitis, there was an extensive tuberculosis of the lymph nodes in the neck, the chest, the axillae, and behind the peritoneum, largely caseous, but not continuous. The advanced caseation indicates that the process in the lymph nodes was old and probably primary.

6. Man, aged 18, had bronchitis and 'glands' in childhood, and died of tuberculosis of the lungs and intestine, with amyloid degeneration. There was an old, general tuberculosis of the lymph nodes in the mesentery and in the hilus of the lungs, along the trachea, as well as in the neck, with calcification and putty-like foci. The process was apparently very old, especially in the abdomen, and in the neck, and was possibly the point of origin of the other localizations.

7. Man, aged 22, died of acute pulmonary tuberculosis and miliary dissemination through rupture of a pulmonary vein. There was a caseous tuberculosis of the cervical nodes, of the nodes in the mediastinum, the hilus of the lungs, along the bronchi, and in the mesentery and behind the peritoneum. The process in the lymph nodes appeared to be the oldest process, especially in the neck and chest, but it was not continuous.

8. Girl, aged 16, who had tuberculous glands which were removed from the neck in childhood, died of miliary tuberculosis. There were also tuberculous intestinal ulcers, tuberculous pleuritis and extensive old tuberculosis of the thoracic lymph nodes with perforation of a pulmonary vein, and extensive tuberculosis of the mesenteric and retroperitoneal lymph nodes, in increasing degree toward the diaphragm, but there was no tuberculosis in the neck. In this case the process in the lymph nodes was primary, according to the history, probably in the neck; from the thorax it appeared to have spread to the abdomen.

9. Man, aged 27, had intestinal tuberculosis, tuberculous peritonitis and pleuritis, with small foci in the lungs, and an old tuberculosis in the lymph nodes of the chest, neck and abdomen, but not continuous. The process in the nodes was oldest and most marked in the chest.

10. Boy, aged 17, had cavernous tuberculosis of the lungs, intestinal tuberculosis, and a general, rather old tuberculosis of the lymph nodes, most marked in the thorax and behind the peritoneum. The process in the lymph nodes appeared to be primary.

11. Woman, aged 19, had tuberculosis of the lungs, larynx, and intestine, with an extensive process in the nodes of chest, neck, and abdomen. The age of, and relation between, the various processes was not clear.

12. Man, aged 22, had had digestive and intestinal disturbances since the age of 10. There was increasing swelling in the neck during the last 3 years, with emaciation. There were tuberculous strictures in the jejunum and caseation of the corresponding mesenteric nodes. There were caseous nodes low down in the neck, also enlarged bronchial nodes, and in 1 apex fibrocalcareous indurations surrounded by recent tubercles. This looked like a case of primary intestinal tuberculosis on which followed a process in the nodes, in the abdomen, and in the neck. While the process in the lung was old, it could hardly explain the extensive changes elsewhere.

13. Girl, aged 15 years, had extensive general lymph-node tuberculosis, especially in the neck, perforation of a bronchus with extension into the lung, and intestinal tuberculosis with ulcer. In this case the lymph-node infection was undoubtedly the oldest. It was most marked in the chest and neck, but not continuous in these regions.

14. Man, aged 27, who had an operation for 'glands' of the neck 3 years past, died of urogenital tuberculosis. There were hard putty-like nodes on the right side of the neck and in the chest and abdomen, largest in the abdomen (size of goose eggs), and tuberculous intestinal ulcers. Undoubtedly a primary lymph-node infection, probably oldest in the abdomen and neck, but without direct connection between these groups.

15. Woman, aged 44, who had 'glands' in childhood, died of enteritis and amyloid disease. There were found intestinal tuberculosis, tuberculous salpingitis, an old focus in 1 lung, and inveterate tuberculosis in the lymph nodes of the chest, neck and mesentery. The last was most likely the oldest lesion, but the relation between the processes in the different groups is not clear.

16. Man, aged 24, died of pneumonia. He had 6 operations for glands in the neck during his 12th and 13th years. Now the cervical nodes were caseous, size of almonds; the nodes in the hilus of the lungs were swollen with scattered fibrous tubercles and there were caseous nodes in the mesentery. There was an old general lymph-node process, but not continuous.

17. Woman, aged 27, with tuberculous abscess in Douglas fossa, was found to have a chronic peribronchial tuberculosis of the lungs, tuberculous ulcers in the intestine, and a genital tuberculosis. In the glands of the neck, chest, and abdomen was an old tuberculosis with caseous and putty-like material, apparently older than the other processes. Whether it represented a continuous lymphatic dissemination could not be determined.

18. Woman, aged 30, of a tuberculous family, had broken down glands in childhood. She died of 'consumption'. There was chronic tuberculosis in the lungs and intestine, tuberculous peritonitis and endometritis, and miliary tuberculosis. The glands in the neck, chest, and abdomen were tuberculous, and the history pointed to an early infection of the cervical glands, but the point of primary infection and the mode of spreading could not be determined anatomically.

19. Woman, aged 27, had 'glands' in childhood, tuberculous nodes in the axilla when 17, presternal tuberculous abscess when 20, spondylitis during the past 7 years, and died of amyloid. Necropsy showed that the tuberculosis in the neck had subsided completely, while in the chest and abdomen there were large caseous nodes. There is a possibility in this case that the infection spread from the cervical nodes.

20. Man, aged 36, died of miliary tuberculosis and meningitis. Besides a small pulmonary focus there were large masses of caseous lymph nodes in the neck, axillae, chest, and abdomen, and a tuberculous ulcer in the cecum, the process in the lymph nodes being apparently continuous.

21. Girl, aged 18 years, had tuberculous peritonitis and pleuritis with caseous foci in the lungs, and thoracic and retroperitoneal lymph nodes. The process in the lymph nodes could hardly be regarded as secondary to the pulmonary process.

22. Woman, aged 19, with a small focus in the right lung, tuberculous salpingitis, peritonitis, pleuritis, and pericarditis (commencing). There were caseous nodes in the chest, neck, and abdomen, the process being apparently independent in each group.

23. Girl, aged 18 years, whose father was tuberculous, had pleuritis 2 years past. There were extensive pulmonary involvement with cavities, tuberculosis of the larynx and intestine, and walnut-sized caseous masses in the neck, chest, and abdomen, showing an extensive and old lymph-node process.

24. Woman, aged 22, had tuberculosis in the cecum and colon, and a general lymph-node tuberculosis of long standing. The intestinal ulcer hardly sufficed to explain the process in the lymph nodes except perhaps by hematogenic dissemination from secondarily affected mesenteric nodes. On the other hand, infection may have taken place along different routes either simultaneously or at different periods.

25. Man, aged 24, died with cerebral symptoms due to a large tuberculous mass in the brain. There were tuberculosis of the lungs with small cavities, tuberculous pleuritis and peritonitis, and an extensive tuberculosis in the lymph nodes in the neck, chest, and abdomen, those in the thorax being most markedly involved and apparently the seat of the oldest changes.

26. Girl, aged 15 years, whose mother was tuberculous, had 'glands' which suppurated. She died of tuberculous meningitis. Besides meningitis, there were found an old pulmonary tuberculosis with scattered caseous and calcified nodules, a chronic ileocecal tuberculosis, and a general lymph-node tuberculosis of chronic character, as shown by calcification and caseation with softening.

Beginning in the neck and extending continuously downward was a series of nodules, bean-sized and larger, partly caseous, which were directly connected with similar nodules in the chest, along the spinal column, and in the hilus, as well as along the bronchi; in the latter place the nodules were completely calcified. The retroperitoneal nodes from the diaphragm down to Poupart's ligament were caseous and as large as walnuts; along the psoas muscles were softened masses with yellowish green pus. This seemed to be a typical example of a chronic general lymph-node tuberculosis, originating early in childhood, in which there was direct communication between the various groups involved. The old pulmonary process may have been of hematogenous origin and the intestinal tuberculosis pointed to a primary infection through the digestive tract.

27. Woman, aged 65, who died of chronic nephritis, had a mass of swollen glands with putty-like contents under the right side of the tongue, also along the trachea and along the aorta, the chest, and the abdomen. No connection between these different groups could be found.

28. Man, aged 25, besides tuberculous peritonitis showed tuberculosis in the lymph nodes, in the mesentery and in the chest, also a fresh tuberculous pleuritis. There was a small caseous focus in 1 lung and fresh tuberculous nodules in the cervical lymph nodes clearly of more recent date than the process in the chest and the abdomen.

29. Man, aged 24, who died of tuberculous meningitis, presented large caseous lymph nodes in the chest, in the supraclavicular regions, and also along the aorta behind the peritoneum, the process apparently having spread from the thoracic nodes both upward and downward.

30. Girl, aged 17, died of pulmonary tuberculosis. She had had enlarged glands in the neck for many years. In addition to a rather acute cavernous process in the lungs were found large caseous nodes in the neck, along the trachea, and in the hilus of both lungs, as well as in the hilus of the liver and in the omentum minus, the process being oldest in the neck. The infection of the lung may be regarded as secondary.

31. Man, aged 22, had tuberculosis of the knee, and amyloid degeneration. There was an extensive old lymphatic tuberculosis beginning high in the neck and extending continuously down into the inguinal regions, the masses being as large as hen's eggs, hard and caseous. A softened mass in the posterior mediastinum had developed into a tuberculous abscess with erosion of the spinal column. A mass behind the peritoneum had grown fast to the stomach with perforation of the mucous membrane and the formation of a large ulcer with undermined edges and caseous spots in the bottom. There were also a cavernous pulmonary tuberculosis and tuberculous intestinal ulcers. In this case the process in the lymph nodes, which appeared to be continuous, clearly was of the longest standing.

32. Man, aged 25, with 'glands' in the neck since very young, had tuberculous meningitis. There were an extensive old lymph-node tuberculosis with marked caseation from high up in the neck down through the chest and abdomen, and into the inguinal regions, miliary tuberculosis, tuberculous ulcers in the intestines, and a small caseous area in the lung. This was undoubtedly a primary general lymph-node tuberculosis.

33. Man, aged 25, had 'glands' in the neck since childhood; in the summer of 1915 a swelling in the left side of the neck appeared, which grew steadily. In 1916 the diagnosis malignant granulomatosis was made; there was pigmentation in the skin of the face and the hands, and a considerable swelling



on both sides of the neck in the form of hard, solid masses; there were also signs of trouble in the lungs. The patient died in April, 1916. On both sides of the neck were conglomerate masses of enlarged lymph nodes, the largest above and behind, the smallest below in direct connection with similar masses along the trachea and in the hilus of the lungs. The masses were hard, homogeneous, and yellowish white on cut surface. The retroperitoneal lymph nodes were changed in the same way, being largest under the diaphragm and decreasing in size downward along the aorta and the iliac arteries. In the lungs were conglomerate masses of tubercles, especially large and numerous in the upper lobes; 1 such mass had softened and pyopneumothorax had resulted; there were 2 large tuberculous masses in the kidneys. The history and necropsy point to an old process in the lymph nodes which became almost general; possibly it began in the neck in childhood and extended by continuity along lymph vessels to the chest and the abdomen; the lesions in the lung may well have been hematogenic as were those in the kidney.

On reviewing these cases of general lymph-node tuberculosis in adults the question at once arises, how do they originate? Do they arise at the same time or at different times, through infection by different paths? Do they spread from one place to other places by lymph vessels or blood vessels? What are their effects, especially with reference to secondary pulmonary tuberculosis or other fatal localizations?

Undoubtedly, many cases arise from invasions along different paths and probably often also at different times. This is especially true when the affection of the lymph nodes is incomplete, even when present in the neck as well as in the chest and abdomen, that is, discontinuous; in the next place, when the affection clearly is of different age in different places, that is to say, has ceased to advance in one place, for example, in the neck, while there are fresh tubercles in another place. Such conditions may be quite distinctly recognizable when the general lymph node affection is discovered incidentally and is relatively easily analyzed. The source of infection may be clear, such as tuberculosis in the father or mother, so that repeated infection may have taken place easily. There are many cases of this kind, but they do not form the majority of the cases of general lymph-node tuberculosis in the strict sense. These appear somewhat differently macroscopically — one finds, in brief, a generally disseminated, apparently continuous tuberculous enlargement of the lymph nodes everywhere, at all events in all the larger groups which generally are examined; and usually the process is of long standing, that is to say, the lymph nodes are considerably enlarged, usually hard, solid, more rarely soft, in which case the disease assumes more acute forms with fever, anemia, etc., with here and there calcareous infiltrations, but especially caseation in high degree; the masses vary, being sometimes even as large as hen's eggs



and even larger, as for instance in the retroperitoneal space, the hilus of the lungs, or high up in the neck.

In the neck the lymph nodes are usually largest above and behind, beneath and behind the lower jaw, diminishing as one goes downward, but present lower down to the clavicular and suprascapular fossae and at superior thoracic aperture, and also often in the axillae. In the chest, the swelling extends along the whole trachea apparently in continuity with the swelling in the neck, but with increasing size as one goes downward, the largest nodes being found at the bifurcation of the trachea and in the hilus of the lungs; the enlarged bronchial nodes decrease steadily toward the lungs. In the mesentery may be enlarged nodes, with or without ulcers in the intestine, but usually not of any great size; retroperitoneally there may be many large swellings, along the aorta, especially behind the stomach and pancreas; in the hilus of the liver and of the spleen the masses may be large also; they may extend along the aorta to the diaphragm, apparently directly continuous with the masses in the lymph nodes of the chest, along the trachea and esophagus; downward they extend along the iliac vessels to the inguinal region, but decreasing in size.

The question now arises as to how the infection took place in the cases in which the whole lymph node system is affected and apparently continuously. It is close at hand to conclude that it is a continuous extension along the lymph vessels from a point of origin in some group of lymph nodes. Thus it might be an extension from a primary tuberculosis in the lymph nodes of the neck, in favor of which would be the information frequently given in the history that, as a child, the patient had 'glands of the neck' with marked swelling and often suppuration with sometimes repeated operations. Such information was given in 12 of the 32 cases here considered. The direct connection between the lymph nodes of the neck and the chest and of those above and below the diaphragm would also seem to favor the view that it is a primary cervical localization. Within each principal localization (neck, chest, and abdomen) the extension as a general rule would seem to be a continuous one, for example, from the hilus of the lungs out along the bronchi. Is there really such mode of communication that it is possible to have the direct connection and extension asserted especially by Weleminsky?<sup>4</sup> In this connection it may be pointed out that the lymph current frequently is reversed in pathologic processes in a particular

<sup>4</sup> Fourth International Congress on Tuberculosis, Rome, 1912.

region. Furthermore, that there is more or less analogy in the extension of malignant tumors, as for instance, the extension of carcinoma apparently continuously from the abdomen into the chest and from the chest into the neck, at any rate to the supraclavicular lymph nodes (Most).

The majority of those who have studied this problem, however, have reached the result, which harmonizes better with the anatomic facts, that every organ and every organ system has its own lymphatic system which communicates with other systems only on the borders and through comparatively small channels (Most, Kitimura, Beitzke, Hart, E. Albrecht). It is conceded, which is in harmony with experimental results, that the lowermost lymph nodes in the neck may be infected from the chest, and that the abdominal, especially the retroperitoneal, may be infected from the chest, and vice versa (H. Albrecht); but a general extension from a single point is denied by most writers.

In 1905 I argued in favor of this sort of continuous extension as frequent and important in adults, although far from present in every case of this kind.

Tendeloo,<sup>5</sup> who has done much work in this field, consistently champions the frequency of lymphogenic extension of tuberculosis, both in the case of tuberculosis in different groups of lymph nodes and in the lungs, kidneys, etc.

Recently Straub<sup>6</sup> believes he has demonstrated that tuberculosis often spreads along the lymph vessels, especially in the abdomen, and from lymph nodes there to the spleen and the liver; furthermore, that a communication takes place between the retroperitoneal glands and the thoracic glands and thence to the lungs; that a retropharyngeal infection may spread not only to the uninvolved side of the neck, but also to the superficial cervical nodes and the peritracheal nodes from which the bronchial nodes in the lungs may become infected, and also the peripancreatic nodes; also that the infection in the neck may travel along the deep lymph channels along the anterior surface of the spine down into the abdomen and then extend to other groups of lymph nodes.

It would seem that this mode of extension still should receive consideration, especially in the case of adults, because it is in harmony

<sup>5</sup> München. med. Wchnschr., 1905, 52, p. 988. Ibid., 1907, 54, p. 105. Handbuch d. Tuberk., 1914, 1, p. 78. Wien. Med. Wchnschr., 1915, 65, p. 321.

<sup>6</sup> Ztschr. f. klin. Med., 1916, 82.

with what we have learned as to reversal of the lymph stream under pathologic conditions and the establishment of communication between adjacent districts.

There are, however, also recent investigations which point in a somewhat different direction and indicate that hematogenic infection of lymph nodes is frequent and important in adults. The investigations of the frequency and kind of tuberculosis in childhood by Ungermann,<sup>7</sup> de Besche<sup>2</sup> and myself<sup>1</sup> also point in this direction, and emphasize the frequency of infection in several groups of lymph nodes even in latent tuberculosis.

Oehlecher also believes on the basis of experimental studies that the organs of the chest are regularly infected by way of the blood from other organs, and Selter<sup>8</sup> made experiments on inhalation by animals of small doses, which led him to conclude that the bacilli quickly enter the blood and localize in different places, such as the lungs, bronchial nodes, etc., whatever the point of invasion may be.

At all events it would seem that the hematogenic origin of general lymph-node tuberculosis merits much consideration. The correct explanation of many cases of extensive tuberculosis in the lymphatic system would seem to be that one or more groups of nodes become infected, whereon the process spreads from node to node in that group and eventually to internal organs, thence indirectly to the corresponding lymph vessels and nodes or directly to lymph nodes, which clearly appear to have a distinct affinity for tuberculosis. Experience shows that in childhood, in which the cases here considered most frequently occur, the infection spreads very easily, especially to the blood and blood vessels. Marked infection and rapid extension would give rise to general lymph-node involvement, which apparently is continuous and simultaneous. This explanation is especially applicable to tuberculosis early in life (as is shown in investigations by Ungermann,<sup>3</sup> de Besche,<sup>2</sup> and myself<sup>1</sup>), and probably also to many cases of extensive and general lymph node tuberculosis in adults.

The question arises why tuberculosis in certain cases does not stop and is not arrested and cured, but relentlessly and steadily advances. The explanation might be the virulence of the bacilli. It is true that probably most instances of this kind of tuberculosis are caused by bacilli of the human type and the frequent statements in the history that a parent had tuberculosis certainly points in this direction, but as yet we do not know anything very definite about the different actions

<sup>7</sup> *Tuberk.-Arb. a. d. k. Gsmdhtsamte*, 1907, 7.

<sup>8</sup> *Deutsch. med. Wehnschr.*, 1916, 42, p. 77.

of tubercle bacilli of the bovine and of the human type, even though it appears to be true that the bovine bacilli as a rule are not especially virulent. It would perhaps be more reasonable to look for the explanation in repeated infections in connection with a lowered resistance.

What then is the consequence of this general infection of the lymph nodes? As pointed out, tuberculosis is the cause of death in the majority of cases, associated with various secondary localizations. Miliary tuberculosis or tuberculous meningitis developed in one fifth of all the cases, tuberculosis of the intestinal tract in also one fifth of all the cases; tuberculosis in the genitalia, the serous membranes, bones, and joints all occurred, although rarely a general marasmus; chronic pulmonary tuberculosis developed in 12 of this series of 30 fatal cases, that is, 40%. Transition forms or mixtures with leukemic conditions and lymphosarcoma may also be encountered.

Again the question arises whether the pulmonary tuberculosis is secondary to the old lymph node process or whether it perhaps is due to a new infection of the lungs by inhalation of tubercle bacilli. My opinion is that most of these cases of tuberculosis of the lungs are derived from the tuberculosis in the lymph nodes either by direct perforation into the lungs, as seen so commonly in children, or due to a hematogenic infection for which there is abundant opportunity in the course of years.

This leads us to a problem of great interest just now and of great practical importance, namely, whether pulmonary tuberculosis in adults is due to a tuberculous infection in childhood, a continued hematogenic infection or autoreinfection, or on the other hand to a new infection during the adult period. The first possibility has been championed by Andvord in Norway and Römer, Much, and Hamburger in Germany, on the basis of the frequent and regular infection in childhood, which is assumed to have a relatively immunizing effect, which in turn has been thought to influence the character of tuberculosis in adult life.

I intend to limit the discussion to the principal points of the investigations in this field and especially to the views advanced by Andvord.<sup>9</sup> In his last article he claims that in about two thirds of all cases infection takes place in childhood and that tuberculosis of the lungs, manifest as well as latent, in adults must be traced back to childhood infection. At least one fourth of all adult tuberculous patients have been 'scrofulous,' and the lung process is a further development of the early infection, probably most frequently by way of the blood. The point

<sup>9</sup> Norsk Mag. f. Lægevidenskaben, 1912, 73, p. 1609.



of departure is most often an old tuberculosis in the lungs and bronchial nodes, probably originally introduced by way of the air. In districts and town in which tuberculosis has existed for generations only 20-30% of the cases of fatal tuberculosis in adults are acute primary infections; in 80-70% the infections may be assumed to have arisen in childhood, but the majority of the early infections are mild, only 10-5% cause later a fatal pulmonary tuberculosis.

From the clinical point of view, Tillisch has attempted to solve the same question by a study of 841 patients of Grefsen Tuberculosis Sanatorium. In about 20% there had been exposure to infection in childhood, while according to the history about 80% were infected outside the home. Clinically demonstrable indications of an infantile infection was found only in 29% of the 20%, but the other patients, whose history gave no information as to the source of infection and who had symptoms of tuberculosis in childhood, increased the number of those who had had tuberculosis early in life to 156, that is, 18½% of 841 tuberculous patients. The latency, however, was often very long, so that it is difficult to understand the connection. The results of the work by Tillisch indicate, however, that an auto-infection must be assumed in some cases while in other cases an infection from the outside or reinfection in adult life must be assumed.

Tillisch holds that his experience in regard to the dependence of a certain amount of pulmonary tuberculosis in adults on infection in childhood is supported by the history of the cases, and also the fact that tuberculous children often die of tuberculosis. He expresses regret that the study of the pathologic anatomic changes have not supplied any evidence in favor of the origin of tuberculosis of the lungs in adults in infantile infection, but he overlooks my treatment of this question.<sup>1</sup>

Of 558 postmortem examinations in adults in 1901-1902, there were 124 deaths from tuberculosis, that is, 22.2%. There were 30 cases with primary extensive lymph node infections, death resulting from tuberculosis in other organs (24% of the fatal cases of tuberculosis), the point of origin being most often the bronchial lymph nodes or a general lymphatic process, not rarely the cervical or mesenteric glands. On the basis of this result, I estimated that 15-20% of all cases of pulmonary tuberculosis in adults arose secondarily, presumably by way of the blood, from other organs, especially the lymph nodes. I have now collected the reports of cases for 1904-1915, 2911 postmortems with a tuberculous death rate of 14.7%. There were 219 cases of death from tuberculosis of the lungs in adults, of which in 39,



that is, 18%, the anatomic picture clearly pointed to a tuberculous infection in childhood. Most often there was an old primary tuberculosis of the lymph nodes with various other internal localizations, in about 50% a general lymph-node tuberculosis (16 cases), tuberculosis of the bronchial nodes (8 cases), of the cervical nodes, the cervical and bronchial nodes, and rarely in the thoracic and abdominal nodes. In addition, primary old tuberculosis in lymph nodes was frequent, especially the general, in which there were found at the same time small foci in the lungs, healed pleuritis, etc., but in which the fatal localization was different.

Hence the figure reached from purely anatomic consideration corresponds to my earlier observations and also fairly closely to the results obtained by Tillisch from data of a different kind.

The question might be raised whether the tubercle bacilli which for many years have been present in latent forms in the lymph nodes preserve their virulence so that they actually give rise to infection. My experiments with inoculations in guinea-pigs with resulting fatal tuberculosis are comparatively few, but all indicate that the virulence is retained unchanged. Such was the result reached also by Bugge<sup>11</sup> from his numerous inoculations of old tuberculous foci in the lungs and bronchial nodes. It would seem then that in about one fifth of all cases of pulmonary tuberculosis in adults one can demonstrate anatomic changes which originated early, probably most often in childhood, and which may have been the source of later infection, eventually of the process in the lungs — hence of autogenous or endogenous infection or reinfection. If this is the case, does it contraindicate the modern view that most tuberculosis late in life, for example, in the lungs, kidneys, bone, and the joints, has the same origin, especially because earlier infections in childhood are assumed to have left behind a relative immunity or increased resistance to new infection? This would probably be a hasty and incorrect conclusion because it is only tuberculous infection with marked and advanced anatomic changes that we have considered; the milder latent infections of childhood, demonstrable by systematic inoculations of lymph nodes or by tuberculin tests, which are present in most children, are not included, and there is no ground to doubt that such infections also influence the organism, possibly in the way of increased resistance. In regard to this point these investigations do not furnish any information and consequently I do not now wish to consider further the Andvord-Römer hypothesis.

<sup>11</sup> Undersökelse om lungetuberkulosens hyppighet og helbredelighet, 1896.

# COMPARISONS OF THE RATE OF GAS-PRODUCTION BY CERTAIN BACTERIA IN RAW AND IN PASTEURIZED MILK \*

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In most of the work concerning the activity of bacteria in raw milk as contrasted with that in heated milk it has been found that there is a real suppression of the rate of multiplication in the raw during the first few hours after milking. However, the explanation of the cause of this phenomenon has differed widely. The method of determination of suppression of bacteria has generally been the plating of the sample at regular intervals of time, using the plate count as the basis for arriving at conclusions. A number of the ablest bacteriologists since 1890 have contributed to this subject.

The earlier workers believed that milk like blood contains protective bodies. Because of this view a milk diet was often advised in some diseases. But it was soon discovered that this much talked-of protective property is a minor factor in dealing with contamination of market milk. Later it was held that raw milk does have an inhibitive influence which modifies its germ content considerably. Some state that pasteurization (60 C. for 30 minutes) partly removes this inhibitive influence, and they hold that this fact demands consideration in the handling of pasteurized market milk.

## OBJECT OF EXPERIMENT

In the study of bacteria in nature, we are often impressed with the fact that some of the most extensive and most powerful forces are those which suppress rather than annihilate. This seems to be true concerning bacterial action. In the case of nonpathogenic bacteria we are, as a general rule, more concerned with their suppression than with their extermination. For instance, it may be true in the case of fresh milk that while its germicidal action may be weak or more likely actually absent, its suppressive influence on bacteria may be an important consideration in the use of fresh milk as food.

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TABLE 1  
RESULTS OF MILK FERMENTATIONS

Fermen- tation Series Number	Date	Milk Used, C.c.	Number Germs per c.c. in Milk Before Inoculation	Kind of Organism Used for Inoculation	Number of Organisms Used for Inoculation
6	4/16	500	312	B. coli	500,000,000
7	4/16	350	627	B. coli	11,470,000,000
8	4/16	500	316	B. coli	840,000,000
14	11/16	22	16,100	B. aerogenes	20,000,000
15	12/16	22	18,260	B. aerogenes	20,000,000
16	12/16	22	1,140	B. aerogenes	20,000,000
17	12/16	22	1,720	B. aerogenes	20,000,000
18	12/16	22	5,565	B. aerogenes	20,000,000
19	12/16	22	3,500	B. aerogenes	20,000,000
20	12/16	22	6,016	B. aerogenes	20,000,000
21	12/16	22	3,515	B. aerogenes	20,000,000
22	12/16	22	9,150	B. aerogenes	20,000,000

For the purpose of determining the presence of or defining the limits of such an inhibiting influence in raw milk and its reduction or loss in the same when heated, the use of parallel samples for comparisons lends itself exceptionally well. However, this method of procedure has been used very little. Most workers have turned their efforts in this direction with the object in view of either proving or disproving the presence of a germicidal action.

In using aliquot samples of raw and pasteurized milk inoculated with equal amounts of the same pure culture of gas-forming bacteria and subjecting them to the same conditions, we have a test that enables us to detect certain kinds of changes which cannot be equaled for delicacy by chemical analysis. We are employing here biologic measurements, and differences not chemically appreciable become important when interpreted in terms of growth and function.

In this paper it is not the intention to try to explain in any case differences between the physiologic activities of bacteria in raw and in heated milk by naming a specific cause or causes, but simply to determine the presence of differences with the idea of obtaining thereby some rough indication of the kind of factors dealt with.

Among the physiologic activities of micro-organisms some of the more easily measured are multiplication, gas-production, acid-production, alkali-production, and peptonization. Of these, some work has been done toward the measurement of differences in rate of multiplication of certain bacteria in raw and heated milk. The results given here take up mainly measurements of gas-production by bacteria in raw and in heated milk.

TABLE 1—*Continued*  
RESULTS OF MILK FERMENTATIONS

Temperature of Incubation, C.	Total Hours of Fermentation	Total Gas Formed in Triplicate Tubes in Raw Milk			Total Gas Formed in Triplicate Tubes in Pasteurized Milk		
		1	2	3	1	2	3
37	16	11.4	8.5	10.3	22.4	22.7	20.7
37	17	19.7	19.9	22.4	25.8	26.0	35.1
37	19	17.5	15.8	16.2	24.1	26.7	22.3
37	72	5.5	6.9	7.1	11.5	10.0	9.6
37	72	6.5	7.4	7.0	9.6	8.5	8.2
30	72	7.2	6.8	5.5	7.4	7.0	8.6
30	72	5.0	5.6	6.1	6.3	5.2	7.2
30	72	6.2	7.9	6.8	9.6	8.2	8.1
30	72	3.8	4.7	5.3	7.3	7.4	7.2
30	72	2.8	4.4	3.5	3.8	5.4	6.9
30	72	7.0	7.4	5.6	12.8	10.5	11.6
30	72	4.8	6.5	6.3	7.8	11.7	8.0

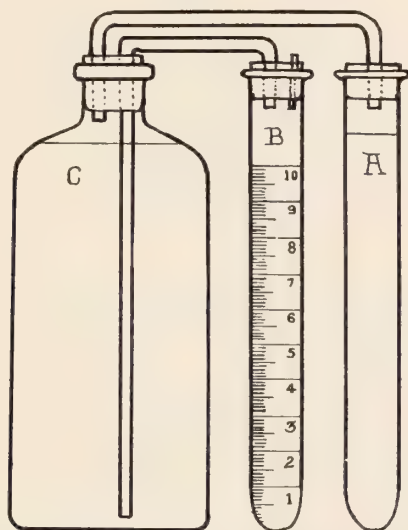


Fig. 1.—Apparatus for fermentation, 3 of which are used in obtaining triplicate results on a single fermentation. A, fermenting liquid; B, graduated test tube for measuring displaced water; C, overflow bottle containing water.

## METHOD

In the comparisons of physiologic activity of bacteria in milk all factors were the same with the exception that part of the milk was raw and part was pasteurized (60 C. for 30 minutes).

- (1) The milk used was divided into 2 parts (a) pasteurized, and (b) raw.
- (2) Triplicate samples of (a) and (b) were inoculated with an equal number of gas-producing organisms using the water suspension method in inoculating quantitatively. Great pains were taken to bring all samples to the same temperature before inoculation.
- (3) All samples were incubated at the same temperature.
- (4) The amount of gas was determined at the end of each hour.

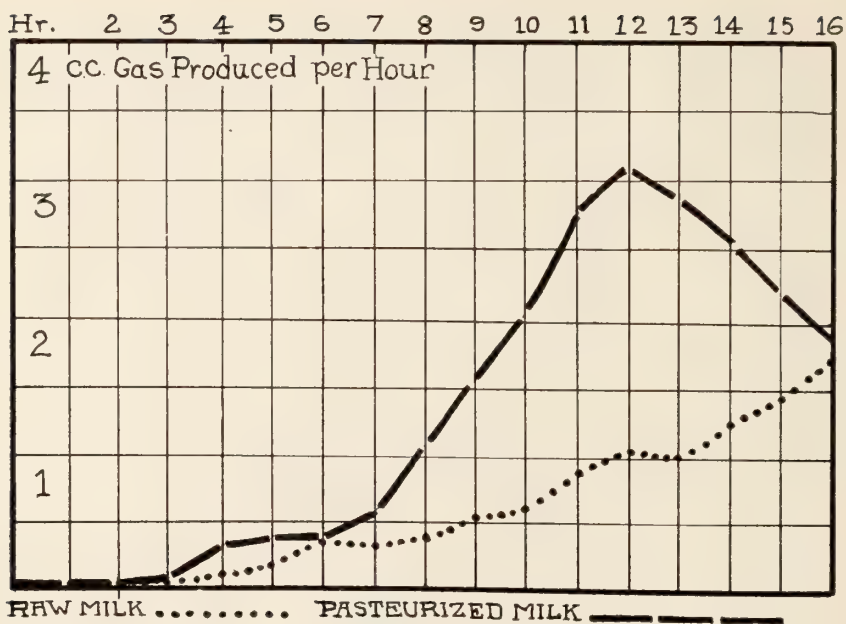


Fig. 2.—Fermentation 8.

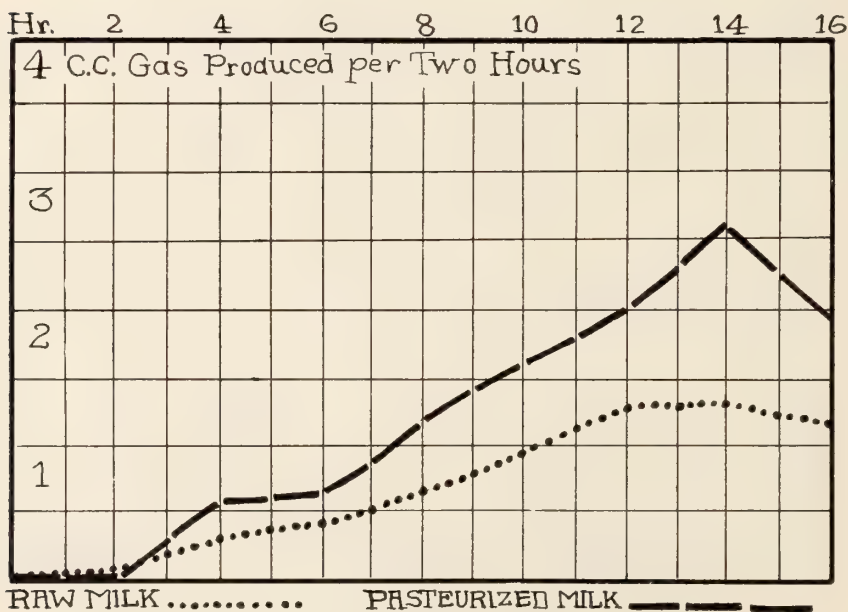


Fig. 3.—Fermentation 21.



TABLE 2  
AVERAGE GAS PRODUCED PER HOUR IN TRIPPLICATE TUBES OF MILK IN FERMENTATION  
SERIES 6, 7, AND 8

Hour	Fermentation					
	Series 6		Series 7		Series 8	
	Raw, C.c.	Pasteurized, C.c.	Raw, C.c.	Pasteurized, C.c.	Raw, C.c.	Pasteurized, C.c.
1	0	0	0	0	0	0
2	0	.06	0	.03	0	0
3	.03	.16	.06	.4	0	.16
4	.16	.3	.1	.23	.03	.2
5	.23	.3	.16	.76	.13	.36
6	.4	.4	.33	.9	.2	.5
7	.3	.56	.23	.166	.16	.83
8	.4	1.16	.46	2.33	.2	.76
9	.53	1.59	1.3	2.6	.4	1.0
10	.63	2.06	1.8	3.26	.56	1.06
11	.86	2.73	2.0	3.6	.83	1.26
12	1.1	3.16	2.23	3.4	1.1	1.43
13	1.0	2.83	2.46	2.83	1.33	1.73
14	1.26	2.63	2.43	2.4	1.5	2.1
15	1.4	2.23	2.33	1.86	1.6	2.26
16	1.73	1.73	2.43	1.36	1.76	2.7
17	.....	.....	2.3	1.2	2.06	2.76
18	.....	.....	.....	.....	2.26	2.6
19	.....	.....	.....	.....	2.33	2.6
	10.06	21.93	20.66	28.96	16.50	24.36

TABLE 3  
AVERAGE GAS PRODUCED PER HOUR IN TRIPPLICATE TUBES OF MILK IN FERMENTATION  
SERIES 18, 19, 20, 21, AND 22

Hours	Fermentation Series 18		Fermentation Series 19		Fermentation Series 20		Fermentation Series 21		Fermentation Series 22	
	Raw, C.c.	Pasteur- ized, C.c.	Raw, C.c.	Pasteur- ized, C.c.	Raw, C.c.	Pasteur- ized, C.c.	Raw, C.c.	Pasteur- ized, C.c.	Raw, C.c.	Pasteur- ized, C.c.
2	.56	.13	0	0	0	0	.13	.1	0	0
4	.4	.66	.3	.3	0	.03	.33	.56	.2	.16
6	.36	.73	.46	.56	.13	.33	.43	.66	.53	.56
8	.66	1.03	.66	.86	.26	.53	.7	1.2	.83	1.06
10	.86	1.1	.86	1.1	.53	.93	.93	1.63	.86	1.86
12	1.26	1.26	.83	1.33	.7	1.23	1.23	2.0	.83	2.06
14	1.43	1.5	.96	1.53	.66	1.56	1.33	2.6	1.2	1.6
16	1.3	1.6	.46	1.2	.8	.86	1.2	1.9	.83	1.63
24	.4	.6	0	.36	0	0	.36	.96	.3	0
48	0	0	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0	0	0
	6.96	8.63	4.6	7.3	3.56	6.03	6.66	11.63	5.86	9.16

TABLE 4  
A TYPICAL SERIES WITH THE COLON BACILLUS; MILK FERMENTATION SERIES 8

Hours	Gas Produced per Hour in Triplicate Tubes of Raw Milk, C.c.				Gas Produced per Hour in Triplicate Tubes of Pasteurized Milk, C.c.			
	1	2	3	Average	1	2	3	Average
0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	.2	.1	.2	.16
4	0	0	.1	.03	.2	.2	.2	.2
5	.2	.1	.1	.13	.4	.5	.2	.36
6	.2	.2	.2	.2	.4	.6	.5	.5
7	.1	.2	.2	.16	.7	1.2	.6	.83
8	.3	.1	.2	.2	.7	1.1	.5	.76
9	.5	.2	.5	.4	.7	1.2	1.1	1.0
10	.6	.5	.6	.56	.9	1.2	1.1	1.06
11	.9	.7	.9	.83	1.2	1.4	1.2	1.26
12	1.0	1.0	1.3	1.1	1.5	1.6	1.2	1.43
13	1.3	1.2	1.5	1.33	1.8	1.9	1.5	1.73
14	1.5	1.6	1.4	1.5	2.4	2.2	1.7	2.1
15	1.9	1.3	1.6	1.6	2.4	2.6	1.8	2.26
16	2.0	1.6	1.7	1.76	2.9	3.0	2.2	2.7
17	2.2	2.1	1.9	2.06	2.7	3.0	2.6	2.76
18	2.5	2.4	1.9	2.26	2.5	2.6	2.7	2.6
19	2.3	2.6	2.1	2.33	2.5	2.3	3.0	2.6
Total at end of 19 hours	17.5	15.8	16.2	16.5	24.1	26.7	22.3	24.36

TABLE 5  
A TYPICAL SERIES WITH B. AEROGENES; MILK FERMENTATION SERIES 21

Hours	Gas Produced During 2-Hour Intervals in Triplicate Tubes of Raw Milk, C.c.				Gas Produced During 2-Hour Intervals in Triplicate Tubes of Pasteurized Milk, C.c.			
	1	2	3	Average	1	2	3	Average
0	0	0	0	0	0	0	0	0
2	0	.1	.3	.13	0	.3	0	.1
4	.4	.3	.3	.33	.5	.5	.7	.56
6	.4	.6	.3	.43	.9	.3	.8	.66
8	.7	.9	.5	.7	1.4	.7	1.5	1.2
10	1.0	1.2	.6	.93	1.5	1.6	1.8	1.63
12	1.3	1.4	1.0	1.23	1.8	1.9	2.3	2.0
14	1.1	1.4	1.5	1.33	2.6	2.5	2.7	2.6
16	1.6	.9	1.1	1.2	2.5	2.2	1.0	1.9
24	.5	.6	0	.36	1.6	.5	.8	.96
48	0	0	0	0	0	0	0	0
72	0	0	0	0	0	0	0	0
Total at end of 72 hours	7.0	7.4	5.6	6.66	12.8	10.5	11.6	11.63

## NOTES CONCERNING FERMENTATION

Triplicate Smith fermentation tube checks were made with the milk used in each fermentation series here reported and resulted in the absence of gas-formation in each case.

The milk used in Series 6, 7, and 8 was obtained in sterile bottles at the barn at milking time while in the outer fermentation series the milk used was obtained from the cans immediately after milking.

The curves given in Figs. 2 and 3 of the rate of gas-production cannot be compared with curves showing rate of increase of total numbers of organisms, as in the former the curves represent at any given time the gas produced per unit of time, while rate of multiplication curves generally represents the total number of organisms accumulated at any given time. If the curves had been drawn on the basis of the total amount of gas accumulated at the end of intervals of time much greater contrast in the courses of the curves would have appeared.

## CONCLUSIONS

Pasteurization causes milk to become more favorable to the attack of the gas-forming colon bacillus and *B. aerogenes*. These results seem to reinforce the impression long held by many milk men that pure raw milk has a power of resisting changes which the same milk does not possess when pasteurized.

Between raw and pasteurized milk there may be important differences, although chemical analyses may show no appreciable differences.

In view of the fact that milk has its value strictly because of its relation to growth, in studying raw and heated milks, due consideration should be given to delicate biologic tests which utilize growth as the means of producing comparative data.

It should be more generally recognized that pasteurized milk instead of receiving less care than raw milk should receive greater care because of its lessened resistance to many detrimental changes which the appearance of the milk does not indicate. This is especially significant in that, in general, pasteurization has lengthened the period between production and consumption.



# THE TITRATION OF DIPHTHERIA TOXIN IN UNILATERALLY NEPHRECTOMIZED GUINEA-PIGS \*

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Experimental observations on the effect of variation in the elimination of bacterial toxins in an animal on its susceptibility to their toxic effect are entirely lacking. The occurrence of distinctive lesions in the kidneys of animals succumbing to diphtheria toxin (Welch and Flexner,<sup>1</sup> Frothingham,<sup>2</sup> Bailey<sup>3</sup>), as well as the frequent occurrence of nephritis in diphtheria patients indicates the importance of the kidney in the elimination of this poison and that variation in renal functional activity may materially affect susceptibility to diphtheria toxin. The object of the present study was to determine the effect of decreased kidney substance, produced by nephrectomy, on the fatal dose of diphtheria toxin.

The left kidney was removed through a half-inch incision in the posterior and upper left lumbar region. Animals with external wound infections following nephrectomy were discarded. The guinea-pigs weighed between 240 and 300 gm. The control animals were normal pigs, splenectomized pigs, and animals in which a piece of omentum had been removed. In all of the experiments the same toxin was used. It had a standard L + dose of 0.2 c.c. and was obtained from the U. S. Public Health Service at Washington, D. C. The dosage was graduated in the same manner as is customary in the titration of an unknown diphtheria toxin. The urine of most of the animals was tested for albumin shortly before injection of the toxin. The animals were carefully observed for toxic symptoms and examined carefully after death for characteristic hemorrhagic edema and induration about the site of injection, hemorrhagic or congested suprarenals, and focal necrosis of the liver. Owing to the well known susceptibility of guinea-pigs to intercurrent infections and sudden fatal epidemics, pneumonia, pleurisy, ascites, and peritonitis were not regarded as evidence of death due to diphtheria toxin without coincident lesions more characteristic of diphtheria intoxication.

In the 1st series of experiments the effect of the nephrectomy was determined on the M. L. D. The results showed a marked diminution

\* Received for publication March 6, 1917.

<sup>1</sup> Bull. Johns Hopkins Hosp., 1891, 2, p. 107.

<sup>2</sup> Jour. Med. Research, 1914, 30, p. 365.

<sup>3</sup> Jour. Exper. Med., 1917, 25, p. 109.



TABLE 1  
TITRATION OF DIPHTHERIA TOXIN

	Determination of L+ Dose					
	Dose, C.c.	Results				
		Death	Local Reaction	Supra- renal Conges- tion	Liver Necrosis	Miscellaneous
Unilaterally nephrectomized guinea-pigs	0.2	48 hr.	±	±	—	—
	0.18	60 hr.	+	—	—	—
	0.16	72 hr.	+	+	—	—
	0.14	.....	..	..	..	Recovery
	0.12	.....	..	..	..	Recovery
Normal control guinea-pigs	0.2	90 hr.	+	+	—	—
	0.18	.....	..	..	..	Recovery
	0.16	.....	..	..	..	Recovery
	0.14	.....	..	..	..	No symptoms
	0.12	60 hr.	—	—	—	Pneumonia
Splenectomized guinea-pigs	0.2	7 days	—	—	—	—
	0.18	.....	..	..	..	Recovery
	0.16	.....	..	..	..	Recovery
	0.14	.....	..	..	..	No symptoms
	0.12	.....	..	..	..	No symptoms

TABLE 2  
TITRATION OF DIPHTHERIA TOXIN WITH ANTITOXIN

Subject	Number	Weight, Gm.	Weight of Left Kidney, Gm.	Post- Opera- tion	Albumin in Urine
Unilaterally nephrectomized guinea-pigs	162	276	1	24 hr.	—
	159	266	1.34	48 hr.	—
	86	260	1.38	96 hr.	—
	68	244	1.25	9 days	—
	64	268	1.35	14 days	—
	123	220	1.45	21 days	—
	120	325	1.8	30 days	—
Control guinea-pigs*	163	269	....	24 hr.	..
	161	266	....	48 hr.	..
	67	215	....	96 hr.	..
	76	235	....	7 days	..
	54	230	....	14 days	..

TABLE 1—*Continued*  
TITRATION OF DIPHTHERIA TOXIN

Determination of M. L. D.*					
Dose, C.c.	Results				
	Death	Local Reaction	Supra- renal Conges- tion	Liver Necrosis	Miscellaneous
0.01	48 hr.	±	±	—	—
0.008	72 hr.	+	+	—	—
0.006	76 hr.	+	+	—	—
0.005	90 hr.	±	±	—	—
0.004	6 days	++	±	++	—
0.003	90 hr.	—	—	—	Peritonitis local infection
0.01	6 days	—	±	—	—
0.008	76 hr.	+	+	—	—
0.006	90 hr.	+	+	+	—
0.005	.....	..	..	..	Recovery
0.004	5 days	+	+	—	—
0.003	.....	..	..	..	Recovery

\* Control animals were subjected to laparotomy with removal of a piece of omentum.

TABLE 2—*Continued*  
TITRATION OF DIPHTHERIA TOXIN WITH ANTITOXIN

Dose, C.c.	Results					
	Death	Local Reaction	Supra- renal Conges- tion	Liver Necrosis	Weight Right Kidney, Gm.	Miscellaneous
0.16	54 hr.	+	+	—	1.4	Recovery
0.16	52 hr.	+	+	+	1.8	
0.16	60 hr.	+	+	—	1.8	
0.16	73 hr.	+	+	—	1.93	
0.16	76 hr.	+	+	—	2.2	
0.16	66 hr.	+	+	+	1.73	
0.16	.....	..	..	..	...	
0.16	.....	..	..	..	...	Symptoms with recovery
0.16	.....	..	..	..	...	Symptoms with recovery
0.16	86 hr.	+	+	..	...	No symptoms Local infection at tail of pancreas
0.16	.....	..	..	..	...	
0.16	60 hr.	+	+	..	...	

\* Laparotomy with removal of some of omental fat.

in the resistance of the nephrectomized animals as compared with normal pigs. When, however, the operative factor with its coincident traumatism and shock was minimized by the use of controls of splenectomized pigs or pigs in which a piece of omentum had been removed, the difference in the resistance was not so great, though still distinctly in favor of the control animal. This is illustrated in Table 1, which gives a summary of the results of 1 of these experiments in which all of the nephrectomized animals succumbed while 2 of the control animals survived. In this experiment it is also of interest that the nephrectomized animal that received the smallest dose of toxin had a trace of albumin in the urine and died before one receiving a larger dose. In another experiment, 1 of the control animals, the urine of which showed a trace of albumin, also showed a decided susceptibility to the toxin.

The 2nd series of experiments was made to determine the effect of nephrectomy upon the L+ dose. When compared with the normal control animals the increased susceptibility of the nephrectomized animals was very striking. With the elimination of the operating factor by the use of splenectomized animals as controls, the resistance of the nephrectomized pigs was also considerably diminished (Table 1).

A final experiment was made to determine the effect of the post-operative period and of the compensatory hypertrophy of the right kidney on the L+ dose. The data of this experiment are summarized in Table 2. The number of guinea-pigs used includes a series operated on at different intervals, but receiving a constant dose of diphtheria toxin. The control animals were operated on approximately the same date, with removal of a section of omentum. In the 1st place, they show conclusively the increased susceptibility of the nephrectomized animals, all but 1 dying while only 2 of the control animals died, and one of these, No. 54, had an operative infection which contributed to its susceptibility, especially since this was the animal with the longest postoperative period and one that otherwise would be expected to survive all others. In the 2nd place, the data point to the relative unimportance of the time elapsing after the operation (under 3 weeks) on the animal's resistance. Furthermore, the degree of compensatory hypertrophy (ascertained by weight) of the remaining kidney does not bear any consistent relation to the resistance to the toxin. For example, Animals No. 68 and No. 64 showed a compensatory hypertrophy of 39% and 63%, respectively, with no appreciable difference in their

resistance, this in spite of the fact that No. 64 had 5 more days' rest after the operation. In other words, increase of kidney substance alone does not cause corresponding increase of resistance. The recovery of No. 120 suggests, however, that the functional activity of the kidney falls behind its anatomic hypertrophy, but will in time fully compensate. There is also considerable variation in the amount of compensatory hypertrophy that occurs in different animals and, in every instance, it falls considerably, usually less than 50%, short of the combined weights of both kidneys before operation. The hypertrophy occurs mainly within the first few days. It is also evident from the behavior of the control animals that guinea-pigs present considerable individual variation, a fact that would make confirmation of such experiments as these very desirable.

The effect of nephrectomy on the M. L. D. is to lower it. This is probably due to delayed elimination of the toxin through the diminution of kidney substance thus allowing the toxin a longer interval to act on the susceptible cells of the body.

Reduction of kidney substance has a much more decided and pronounced effect on the L+ dose, a fact that is important in many respects. It is well known that the L+ dose is by far the most accurate and reliable index of the action and strength of diphtheria toxin, owing to the elimination of toxoid bodies. The decided reduction of the L+ dose in nephrectomized animals shows that diminution of the functional activity of the kidneys enhances the action of diphtheria toxin and lowers the efficacy of the antitoxins. The exact mechanism of this action cannot be easily proved. It may mean either the interference with the elimination of the toxin, the increased susceptibility of the tissue, or both. Moreover, nephrectomy may bring about the lowered resistance, in fact, must do so, at least in part by partial dissociation of the toxin-antitoxin combination. The L+ dose in the nephrectomized animals is so much reduced that no other explanation can be offered than that the decreased kidney function has increased the affinity for the toxin molecule so much that it is greater for the tissue cells than for the antitoxin molecule with liberation of free antitoxin. In other words, the toxin has a greater affinity for cells of low vitality than for antitoxin. This thus contributes further evidence to the theory advanced and supported by Roux and Vaillard,<sup>4</sup> Buchner,<sup>5</sup>

<sup>4</sup> Ann. de l'Inst. Pasteur, 1893, 7, p. 65.

<sup>5</sup> München. Med. Wchschr., 1893, 40, p. 427.

Calmette,<sup>6</sup> Morgenroth,<sup>7</sup> and Arrhenius,<sup>8</sup> that the toxin-antitoxin combination is an unstable one and can be easily broken down with liberation of the toxin.

The importance of examining the urine of guinea-pigs used for standardization of diphtheria toxin and antitoxin for diminished or diseased kidney function is evident. In routine tests of guinea-pigs, it was found that approximately 2% of normal pigs and 17% of nephrectomized animals showed variable traces of albumin in the urine. In the course of these experiments a trace of albumin has played a very suggestive part in explaining the lowered resistance of 2 animals to the toxin. The wisdom of making titrations of toxins for standardization on a large series of control animals, as is done in some laboratories, is also apparent.

<sup>6</sup> Ann. de l'Inst. Pasteur, 1895, 9, p. 225.

<sup>7</sup> Berl. klin. Wchnschr., 1905, 50, p. 1550.

<sup>8</sup> Immunochemistry, 1907, p. 12.



# COMPARATIVE STUDY OF DIFFERENT ANTIGENS AND OF DIFFERENT TEMPERATURES OF INCUBA- TION IN THE WASSERMANN TEST \*

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## INTRODUCTION

It is well recognized that widely different results may be obtained in complement-fixation tests for syphilis by the use of different antigens and by the employment of different incubation conditions, even with the same antigens. In a previous communication<sup>1</sup> we have reported on Wassermann tests that were performed by 3 methods on 496 identical specimens from 477 patients. In the 1st method a cholesterin-reinforced antigen was employed and the 1st incubation was carried out at 37 C. In the 2nd method a simple alcoholic extract was used as antigen, with incubation also at 37 C. In the 3rd method this latter antigen was again employed, but the 1st incubation was carried out in the refrigerator for 4-24 hours. The last method proved more sensitive in the group of known syphilitics than the other procedures tested. Furthermore, a positive result thus obtained proved to be more trustworthy evidence of syphilis than did positive results obtained with the cholesterinized antigen and 1st incubation at 37 C.

In the present paper we shall present the results of Wassermann tests performed by 6 methods on 501 identical specimens from 457 patients. In the 1st of these 6 methods a cholesterin-reinforced antigen was employed, and the 1st incubation was carried out at 37 C. In the 2nd method a simple alcoholic extract was used as antigen, with incubation also at 37 C. In the 3rd method an antigen prepared from the acetone-insoluble fraction of beef heart, after the method of Noguchi,<sup>2</sup> was used, with incubation also at 37 C. Exact duplicates of these tests were prepared and incubated at 8 C., for 4 hours. Tests 4, 5, and 6 differ from Tests 1, 2, and 3, respectively, only in the temperature and length of time for the 1st incubation.

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<sup>1</sup> Smith, J. Wheeler, Jr., and MacNeal, W. J.: *Jour. Immunol.*, 1916, 2, p. 75.

<sup>2</sup> *Serum Diagnosis of Syphilis and the Butyric Acid Test for Syphilis*, 1911, p. 79.

## PREPARATION OF REAGENTS AND THE TECHNIC OF THE TESTS

The antigens used in this study were all prepared from beef heart. Fat and fibrous tissue were cut away, blood clots were washed out with cold water, and the lean muscle was wiped dry and passed through a meat-chopper. One hundred and twenty grams of the chopped meat were extracted in 1200 c.c. of absolute alcohol at 37 C., with occasional shaking, for 2 weeks. The preparation was then filtered through paper until clear. A portion of the filtrate, measuring 100 c.c., was put aside for use as simple alcoholic extract antigen, designated as B. H. P. (beef heart, plain). To another portion of 100 c.c., 0.4 gm. of cholesterin was added. This mixture was refrigerated over night and again filtered. The filtrate was employed as cholesterin-reinforced antigen,

TABLE 1  
SCHEME OF TITRATION OF THE ANTIGENS

(a) Hemolytic Titration					
Antigen, 10% emulsion.....	0.1	0.2	0.3	0.4	
Salt solution, 0.9%.....	0.7	0.6	0.5	0.4	
R. B. C., 5% suspension.....	0.2	0.2	0.2	0.2	

Incubate 2 sets of tubes, one at 37 C. for 30 minutes and the other at 8 C. for 4 hours and make readings.

(b) Anticomplementary Titration					
Antigen, 10% emulsion.....	0.1	0.2	0.3	0.4	
Salt solution, 0.9%.....	0.3	0.2	0.1		
Complement, 10% .....	0.2	0.2	0.2	0.2	

Incubate 2 sets of tubes, one at 37 C. for 1 hour and the other at 8 C. for 4 hours. Then add 0.1 c.c. of suspension of sensitized red blood cells, incubate at 37 C. for 30 minutes, and make readings.

(c) Specific Antigenic Titration									
Antigen, 10% emulsion.....	...	...	...	...	...	0.05	0.1	0.2	
Antigen, 1% emulsion.....	0.01	0.02	0.05	0.1	0.2				
Very positive serum, 20%.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Salt solution, 0.9%.....	0.3	0.3	0.3	0.2	0.1	0.3	0.2	0.1	0.3
Complement, 10% .....	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Incubate for fixation, one set at 37 C., for 1 hour and the other at 8 C., for 4 hours. Then add 0.4 c.c. of suspension of sensitized red blood cells, incubate at 37 C. for 30 minutes, and make readings.

labeled B. H. C. (beef heart, cholesterinized). What remained of the original filtrate, approximately 1000 c.c., was used in the preparation of the acetone-insoluble antigen of Noguchi.<sup>2</sup> It was identified as Antigen B. H. A. (beef heart, acetone-insoluble fraction).

Each antigen was titrated for (1) hemolytic effect, (2) anticomplementary action, and (3) specific antigenic property. The titrations were made for each set of incubation conditions, that is, 37 C. for 1 hour and 8 C. for 4 hours.

A type protocol of the titrations of the antigens is shown in Table 1.

When tested according to this scheme, the plain alcoholic extract, Antigen No. 74, B. H. P., was not hemolytic and not anticomplementary at either temperature in the maximum quantities tested. The antigenic titer showed the

smallest amount necessary for complete fixation to be 0.05 c.c. of a 10% emulsion, at 37 C., and 0.2 c.c. of a 1% emulsion at 8 C. The cholesterinized alcoholic extract, Antigen 74 (a), B. H. C., was not hemolytic at either temperature in the maximum quantities tested. It was anticomplementary in dose of 0.4 c.c. at 37 C., and also at 8 C. The antigenic titer proved to be 0.2 c.c. of a 1% emulsion, at 37 C., and 0.1 c.c. of a 1% emulsion at 8 C. The corresponding acetone-insoluble preparation, Antigen No. 75, B. H. A., was not hemolytic at 37 C., nor at 8 C. in the maximum quantities tested. It was anticomplementary in dose of 0.4 c.c. at both temperatures. Its antigenic titer was 0.05 c.c. of a 1% emulsion, at 37 C., and 0.01 c.c. of 1% emulsion at 8 C.

It will be observed that the only differences in the titers occurred in the tests of the specific antigenic property and in the absence of apparent anticomplementary action from Antigen B. H. P. The preparations were all more antigenic at 8 C. than at 37 C. The titrations recorded were those made when the antigens were prepared, in September, 1916. This study was made during September, October and November of 1916 and, occasionally, toward the latter part of the work, the anticomplementary controls on Antigen B. H. C. indicated an increasing anticomplementary titer in that antigen. At such times the antigen was anticomplementary in 0.4 c.c. at 37 C., but in 0.3 c.c. at 8 C. On 1 or 2 occasions, when the hemolytic system was a bit tighter than usual, Antigen B. H. C. was found to be anticomplementary in 0.2 c.c., and partially so in 0.1 c.c. It would, therefore, seem to be advisable to employ a more dilute emulsion of the cholesterinized antigen for use at 8 C. It is quite possible to do so, for the antigenic titer, as has been noted, is higher at 8 C. than at 37 C.

The hemolytic system used was the sheep-rabbit system. The unit of cells was 0.2 c.c. of a 5% suspension of sheep's erythrocytes, repeatedly washed and finally packed for 15 minutes at 1500 r. p. m. The unit of complement was 0.1 c.c. of a 10% solution of fresh, active guinea-pig serum. The unit of amboceptor was determined each time. For this study we employed an amboceptor serum of which the unit was always in the neighborhood of 0.1 c.c. of 1:1000 dilution, that being the quantity which, with 1 unit of complement, effected complete hemolysis of 1 unit of cells in 15 minutes at 37 C. Two units each of amboceptor and complement were used in the tests. This system, it will be observed, is quite sufficiently loose to obviate very largely the danger of non-specific fixation. And yet, as will subsequently appear, we have found reactions in 16 of the 501 tests, or 3.2%, which we feel inclined to regard as false positive reactions (Tables 10 and 11).

The serums to be tested were always heated at 56 C., for 30 minutes, just prior to use.

The tests for incubation at 37 C. and those for incubation at 8 C. were exact duplicates. In each case 4 tubes were used, the contents of which were as follows: Tube 1: patient's serum, 0.04 c.c.; Antigen B. H. C., 0.1 c.c.; complement, 0.2 c.c.; salt solution to make 0.6 c.c. Tube 2: patient's serum, 0.04 c.c.; Antigen B. H. P., 0.1 c.c.; complement, 0.2 c.c.; salt solution to balance. Tube 3: patient's serum, 0.04 c.c.; Antigen B. H. A., 0.1 c.c.; complement, 0.2 c.c.; salt solution to balance. Tube 4: patient's serum, 0.08 c.c.; no antigen; complement, 0.2 c.c.; salt solution to balance. One set of tubes was incubated at 37 C., for 1 hour; the other set at 8 C. (ice-box), for 4 hours. At the end of the period of incubation, in each case, 0.4 c.c. of sensitized red cells was added to each tube and the tubes were again incubated. Those which had been kept in the ice-box were put into a water bath, the initial

temperature of which was 45 C. This was because the tubes and the contents had become so thoroughly chilled that it was necessary to employ a greater degree of heat for them than for the others, to allow for absorption. No further heat was applied during incubation, so that at the end of about 15 minutes the temperature of the bath had fallen to about 37 C. The tests were read when the control tubes showed complete hemolysis, which was usually in 15-30 minutes. A representation of the set-up is shown in Table 2.

TABLE 2  
SCHEME OF SETTING UP THE TESTS

	1	2	3	4
Patient's serum.....	0.04	0.04	0.04	0.05
Salt solution.....	0.3	0.3	0.3	0.3
	B. H. C.	B. H. P.	B. H. A.	
Antigen.....	0.1	0.1	0.1	0.1
Complement.....	0.2	0.2	0.2	0.2
Incubation				
Sensitized cells.....	0.4	0.4	0.4	0.4

#### RESULTS OF THE TESTS

The cases herewith presented may be divided into 2 classes on the basis of history. The known syphilitics make up the 1st class. They gave histories of infection, and most of them had had treatment or were under treatment at the time the tests were made. This class we shall call Group I, and in this group we record 92 reactions in 80 cases. This record is presented briefly in Table 3 and, in greater detail, in Table 4.

TABLE 3  
SUMMARIZED RESULTS OF 92 TESTS ON 80 SYPHILITIC PATIENTS, GROUP I

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	48	52.1	3	3.2	41	44.7
2. Simple at 37 C. ....	24	26.0	1	1.0	67	73.0
3. Acetone insoluble at 37 C. ....	29	31.5	0	0.0	63	68.5
4. Cholesterinized at 8 C. ....	70	76.0	3	3.2	19	20.8
5. Simple at 8 C. ....	59	64.1	4	4.3	29	31.6
6. Acetone insoluble at 8 C. ....	43	46.7	3	3.2	46	50.1

The figures presented in Table 3 demonstrate clearly the greater delicacy obtained by ice-box incubation. At 37 C., antigens B. H. C., B. H. P., and B. H. A. yielded, respectively, 52.1%, 26.0%, and 31.5% of positive reactions, while by the ice-box method, on the other hand, they gave, in the same order, 76%, 64.1%, and 46.7% positive tests.

It should be noted that the simple extract antigen at 8 C., with 64.1% positive, is much superior to the cholesterinized antigen at 37 C., which yielded 52.1% positive. This is a confirmation of 1 of the findings which we have already recorded.<sup>1</sup> The cholesterin-reinforced antigen at 8 C. yields the highest percentage of positive reactions in cases of syphilis. It may be noted that although Antigen B. H. A. is of slightly greater value than Antigen B. H. P. at 37 C., it is much inferior at 8 C.

The 2nd class of cases is composed of those in which there are no histories of syphilis. We shall subdivide this class by a consideration of factors other than the history. It is, of course, manifestly absurd to consider a case nonsyphilitic simply because there is no history of syphilis. It becomes necessary to consider the physical findings, the visible and other evidences of disease. On this basis it is possible to form several subdivisions of this group, the 1st of which consists of those cases which presented lesions typically luetic or which, in the absence of other etiologic factors, are usually considered to be luetic. This group we shall call Group II A. It contains cases which, although not avowedly luetic, are probably luetic. We have placed 24 cases in Group II A and on the serums and cerebro-spinal fluids of these, 27 tests have been made. The results are summarized briefly in Table 5 and presented in greater detail in Table 6.

A comparison of Tables 3 and 5 reveals a remarkable similarity. Although the figures in Table 5 are absolutely much higher than those of Table 3, due, undoubtedly, to the fact that the cases of Group II A had not received antiluetic treatment, the relations among the figures themselves are very much the same. Thus, as in Table 3, although Antigen B. H. C. is superior to Antigen B. H. P. at 37 C., the latter at 8 C. is equal to Antigen B. H. C. at 37 C., but Antigen B. H. C. at 8 C. is best of all, yielding 100% positive reactions.

There seems to be little, if any, doubt concerning the luetic nature of cases in Group II A. This cannot be said, however, of all the cases in the next group, which we shall call Group II B. They were cases with no histories of syphilis nor of antisyphilitic treatment, and in which the physical findings were rather indefinite or, as in 3 of the cases, entirely negative. In each of these cases there was, however, a note of suspicion in the history. Thus, in the case of Specimen 7, in Table 8, the patient was the sweetheart of the patient who furnished Specimen 6, in Table 4. In Case 9 the history indicated a possible



TABLE 4  
BRIEF DATA CONCERNING 92 TESTS ON 80 SYPHILITIC PATIENTS, GROUP I

No.	History of Infection	Treatment	Sex	Age	Manifestations at Time of the Tests
1	16 years ago		♂	37	Mild jaundice and gastric disturbance...
2	14 years ago	606 (VI); Hg (X)	♂	31	Sore throat
3	3 weeks ago		♂	26	Sore on penis
4	1½ years ago	606 (II); Hg; KI	♂	49	Pain in knees
5		606; Hg	♂	23	No manifestations
6	2 years ago	Hg (very little)	♂	21	Tumor of liver
7			♂	34	Ulceration of hard palate
8	20 years ago		♂	42	Old ulcer of tongue
9	7 years ago	Hg (very little)	♂	40	Leukoplakia
10*	2 years ago	Hg (very little)	♂	21	Tumor of liver
11	4 years ago		♂	35	Poor vision; rhinitis
12		606; Hg; KI	♂	39	
13			♂	42	
14†		606; Hg; KI	♂	39	
15			♂	30	No manifestations
16	1904	606 (II); Hg	♂	35	No manifestations
17	1896	Hg; KI	♂	38	Nervousness
18	6 weeks ago		♂	32	Sore throat and rash for 2 weeks
19	7 months ago	606 (I); Hg (X)	♂	23	No manifestations
20	28 years ago	606; Hg	♂	48	No manifestations
21	6 years ago	Hg; KI	♂	30	Pains in legs
22	Acquired from father	606; Hg	♂	10	Optic neuritis; sore throat
23	1904	Hg	♂	38	Sores on face
24			♂	53	Nasal and pharyngeal ulceration
25		606 (VI); Hg (XV)	♂	24	
26			♂	55	
27	35 years ago	Hg; KI	♂	58	Ulcer of leg
28	1914	Hg 2 years ago	♂	22	No manifestations
29	1898		♂	39	
30;		606; Hg; KI	♂	36	
31†		606; Hg; KI	♂	36	
32		606; Hg; KI	♂	36	
33		606 (VI); Hg (XV)	♂	33	No manifestations
34	Rash 3 yr. ago; iritis 2 yr. ago		♂	30	
35		Hg (XVI)	♂	39	
36		606; Hg; KI	♂	41	
37	1913	606; Hg; KI	♂	27	Delayed union, fractured tibia
38		606; Hg; KI	♂	14	
39		606 (VI)	♂	7	
40		606; Hg	♂	33	No manifestations
41		606; Hg; KI	♂	36	
42*		606; Hg; KI	♂	26	
43	1912	606 (IV)	♂	34	No manifestations
44	1906	Hg	♂	40	Tubercular syphilid on neck
45	1896		♂	44	Ulcerating lesions, cheek and neck
46	1896	Hg	♂	38	
47		606 (III)	♂	31	No manifestations
48	1907		♂	35	
49	1909	606 (III); Hg (XL)	♂	28	
50	2 months ago		♂	19	Sore throat; rash
51	1914	606 (V); Hg (XXX)	♂	30	
52		606 (VIII); Hg (XV)	♂	17	Keratitis
53			♂	32	
54			♂	43	Hydrocele
55			♂	32	
56†		Hg	♂	27	Penile chancre, 8 days old; spirochetes found
57			♂	54	Papular lesions, face
58	1905		♂	25	
59		606; Hg	♂	29	
60	March, 1916	Hg (XVI); 606 (VI)	♂	32	Incontinence
61	1906		♂	35	
62	8 months ago	Hg (XXXVII)	♂	40	
63	1912		♂	56	Ulcer of nose, 1 year's duration
64	1901	Hg (1 year)	♂	36	Infected fracture of os calcis; temperature 105
65	1898	Hg	♂	32	
67			♂		

TABLE 4—Continued  
BRIEF DATA CONCERNING 92 TESTS ON 80 SYPHILITIC PATIENTS, GROUP I

Diagnosis	Complement-Fixation							
	At 37 C.				At 8 C.			
	1	2	3	4	1	2	3	4
Lues III	—	—	—	—	++++	++++	++++	—
Lues III	—	—	—	—	++++	++++	++++	—
Lues I	—	—	—	—	++++	++++	—	—
Lues III	—	—	++++	—	++++	++++	++++	—
Inactive lues	++	—	—	—	+	+	—	—
Gumma	+++	—	++++	—	++++	++++	++++	—
Lues III	++++	+++	—	—	++++	++++	++++	—
Lues III	—	—	—	—	—	—	—	—
.....	++++	—	—	—	++++	++++	—	—
Gumma	++++	+++	++++	—	++++	++++	++++	—
Lues III	—	—	—	—	+	++++	++++	—
Paresis	—	—	—	—	++++	++++	—	—
Paresis	—	—	—	—	++++	++++	+	—
Paresis	—	—	—	—	—	—	—	—
Inactive lues	++	—	—	—	++++	—	—	—
Inactive lues	++	—	—	—	++++	++++	—	—
.....	—	—	—	—	++++	++++	—	—
Lues II	++++	—	++++	—	+	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
.....	++++	+	++++	—	++++	++++	++++	—
.....	++++	+	++++	—	++++	++++	++++	—
.....	++++	—	++	—	++++	++++	++++	—
Lues III	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	—	—	—	—
Paresis	++++	++++	++++	—	++++	++++	++++	—
.....	—	—	—	—	—	—	—	—
Inactive lues	++++	—	—	—	++++	++++	—	—
Paresis	++++	—	—	—	++++	++++	—	—
Paresis	—	—	—	—	—	—	—	—
Paresis	++++	—	—	—	++++	++++	++++	—
Paresis	++++	—	—	—	++++	+	—	—
.....	++++	—	—	—	—	—	—	—
.....	++++	—	++	—	++++	++++	++++	—
.....	++++	—	—	—	++++	++++	++++	—
Paresis	++++	—	—	—	++++	++++	++++	—
.....	—	—	—	—	++++	++++	++++	—
Juvenile paresis	++++	++++	++++	—	++++	++++	++++	—
Congenital lues	++++	++++	++++	—	++++	++++	++++	—
.....	—	—	—	—	++++	—	—	—
Paresis	++++	—	—	—	++++	++++	++++	—
Paresis	—	—	—	—	++++	++++	++++	—
.....	—	—	—	—	—	—	—	—
Lues III	—	—	—	—	++++	++++	++++	—
Lues III	—	—	—	—	++++	++++	++++	—
.....	+	—	—	—	++++	—	—	—
.....	+	—	—	—	++++	—	—	—
.....	++++	—	—	—	++++	++++	—	—
Lues II	++++	++++	++++	—	++++	++++	++++	—
.....	—	—	—	—	—	—	—	—
Congenital lues	—	—	—	—	++++	—	—	—
Lues III	—	—	—	—	++++	++++	++++	—
Cerebrospinal lues	++++	++++	++++	—	++++	++++	++++	—
Lues I	—	—	—	—	++++	+	—	—
.....	—	—	—	—	—	—	—	—
Lues III	++++	—	—	—	++++	++++	+	—
.....	++++	++	++	—	++++	++++	+	—
.....	—	—	—	—	—	—	—	—
Tabes	—	—	—	—	++++	+	—	—
.....	—	—	—	—	—	—	—	—
.....	++++	—	—	—	++++	++++	—	—
Lues III	++++	++++	++++	—	++++	++++	++++	—
.....	—	—	—	—	++++	++++	—	—
Paresis	++++	++++	++++	—	++++	++++	++++	—

TABLE 4—Continued  
 BRIEF DATA CONCERNING 92 TESTS ON 80 SYPHILITIC PATIENTS, GROUP I

No.	History of Infection	Treatment	Sex	Age	Manifestations at Time of the Tests
68†	.....	.....	♂	34	.....
69‡	.....	.....	♂	32	.....
70	1908	.....	♂	47	Papillitis in both eyes.....
70†	.....	.....	♂	45	A. R. pupils; difficulty in walking; nocturnal micturition
71*	.....	606; Hg; KI	♂	36	.....
72	1908	.....	♂	28	Ulcer of leg.....
73	.....	Hg (XVI); 606 (IV)	♂	29	.....
74	Husband is luetic	.....	♂	19	Cervical and inguinal adenitis.....
75	.....	Hg; 606	♂	27	.....
76	1911	606 (III)	♂	34	.....
77	.....	.....	♂	?	.....
78‡	.....	606; Hg; KI	♂	36	.....
79*	.....	606; Hg; KI	♂	41	.....
80	.....	.....	♂	47	.....
81‡	.....	606; Hg; KI	♂	41	.....
82‡	.....	606; Hg; KI	♂	41	.....
83	.....	.....	♂	50	.....
84	.....	.....	♂	?	.....
85	1906	KI (1 year)	♂	52	Joint pains, especially at night.....
86	1913	Hg	♂	39	Dizziness; pain in back.....
87	1900	.....	♂	31	Gastric symptoms.....
88*	.....	606; Hg; KI	♂	36	.....
89	6 weeks ago	.....	♂	29	Penile sore; rash; malaise.....
90	1906	606 (VI)	♂	30	Generalized body pains.....
91	.....	.....	♂	33	Swelling of right knee.....
92	.....	.....	♂	42	Iritis.....

\* A simple number indicates blood serum. A number with asterisk indicates a duplicate specimen of blood serum from a patient previously tested. Specimen 10\* was obtained from the same patient as Specimen 6; 71\* as 41; 79\* as 36; 88\* as 30.

† A number followed by this character designates a spinal fluid. The patient's blood serum was usually tested also. The following numbers indicate spinal fluid and blood serum, respectively, from the same patient: 14† and 12; 31† and 32; 69† and 67. Specimens 56†, 68†, and 70† were from patients whose blood serum was not tested.

‡ A number followed by this character designates cerebral fluid. The patient's blood serum was usually tested also. The following numbers indicate cerebral fluid and blood serum, respectively, from the same patient: 30‡ and 32; 42‡ and 41; 69‡ and 67; 78‡ and 71\*; 81‡ and 79\*.

primary lesion in 1911. The patient was then aged 21 and probably did not, at that time, appreciate the gravity of a little ulcer on the penis. In Case 15 it was stated frankly that the husband was syphilitic. In all of the cases in this group, syphilis was seriously considered in the differential diagnosis and in most of them the tests revealed fixation of complement with all of the antigens, both at 37 C. and 8 C.

At first thought it seems rather inappropriate, in a study of the value of the Wassermann reaction, to utilize the results of this test in the classification of cases. However, this study is not especially concerned with the absolute value of the reaction, but rather with the relative values of different methods of performing it, and we think that we may be allowed the liberty of utilizing the evidence furnished by the Wassermann test along with the other signs and symptoms.

TABLE 4—Continued  
BRIEF DATA CONCERNING 92 TESTS ON 80 SYPHILITIC PATIENTS, GROUP I

Diagnosis	Complement-Fixation							
	At 37 C.				At 8 C.			
	1	2	3	4	1	2	3	4
Cerebrospinal lues	—	—	—	—	++++	++++	—	—
Paresis	++++	++++	++++	—	++++	++++	++++	—
Taboparesis	++++	++	++	—	++++	++++	++++	—
	+	—	—	—	++++	++	—	—
Paresis	—	—	—	—	—	—	—	—
	++++	++++	++++	—	++++	++++	++++	—
	++	—	—	—	++	—	—	—
	++++	++++	++++	—	++++	++++	++++	—
	++	—	—	—	++	—	—	—
	++++	—	—	—	++++	—	—	—
Paresis	—	—	—	—	++++	++++	++++	—
Paresis	—	—	—	—	—	—	—	—
	++++	++++	++++	—	++++	++++	++++	—
Paresis	—	—	—	—	—	—	—	—
Paresis	—	—	—	—	++++	++++	++++	—
Paresis	—	—	—	—	++++	++++	++++	—
	++++	++++	++++	—	++++	++++	++++	—
	+	—	—	—	++++	—	—	—
	++++	—	—	—	—	—	—	—
	++++	++	++	—	++++	++++	++++	—
Paresis	++++	—	—	—	++++	—	—	—
Lues	++++	++++	—	—	++++	++++	++++	—
	++++	++++	++++	—	++++	++++	++++	—
Charcot's joint	++++	++++	++++	—	++++	++++	++++	—
	++++	++++	++++	—	++++	++++	++++	—

Table 7 presents, in summarized form, the findings in Group II B. Brief data in regard to each test are given in Table 8.

What has already been said of Tables 3 and 5 may be said, also, of Table 7. Antigen B. H. P., at 8 C., is again quite as good as Antigen B. H. C., at 37 C., but Antigen B. H. C., at 8 C., is the best, yielding 92% positive reactions.

TABLE 5  
SUMMARIZED RESULTS OF 27 REACTIONS IN 24 PATIENTS, EVIDENTLY SYPHILITIC, GROUP II A

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	25	92.5	0	0.0	2	7.5
2. Simple at 37 C. ....	18	66.6	1	3.8	8	29.6
3. Acetone insoluble at 37 C. ....	21	77.7	0	0.0	6	22.3
4. Cholesterinized at 8 C. ....	27	100.0	0	0.0	0	0.0
5. Simple at 8 C. ....	25	92.5	0	0.0	2	7.5
6. Acetone insoluble at 8 C. ....	24	88.7	1	3.8	2	7.5

For convenience, all of the cases in Groups I, II A, and II B may be considered together as cases certainly or probably of syphilis. The summarized results of these tests, considered together, are shown in Table 9.

TABLE 6

BRIEF DATA CONCERNING 27 TESTS ON 24 PATIENTS EVIDENTLY SYPHILITIC, GROUP II A

No.	Sex	Age	Manifestations at Time of the Tests	Diagnosis
1	♀	21	Eruption on palms of hands.....	Lues III
2	♀	16	Dyspnea; enlarged heart; murmur.....	Aortic regurgitation
3	♀	3½	Pain and disability, right arm (sister luetic).....	Luetic osteoperiostitis
4	♀	29	Mass in nose.....	Gumma
5	♀	21	Discharging sinus of jaw.....	Chronic osteomyelitis
6	♀	26	Sore on cheek, 8 months' duration.....	Lues III
7	♀	21	Sore throat.....	Lues II
8	♀	1 mo.	Fissures about mouth and anus; malnutrition..	Congenital lues
9	♀	25	Chronic pharyngitis.....	Lues
10	♀	28	Headache; tumor of frontal bone.....	Gumma
11	♀	29	Lesion on side of nose, 2 months' duration.....	Lues
12	♀	36	Lesions on both elbows.....	Lues
13	♀	55	Chronic arthritis with atrophy of bones of ankle	Lues
14	♀	32	Tumor of tonsil.....	Gumma
15	♀	34	Neuralgia.....	Lues
16	♀	33	Sore throat.....	Gumma of larynx
17	♀	11	Acute meningitis.....	Meningeal lues
18	♀	23	Sore throat.....	Lues II
19	♀	26	Sore throat, 3 weeks' duration.....	Lues II
20*	♀	55	See 13.....	.....
21†	♀	11	See 17.....	.....
22	♀	41	Indurated areas, left calf, tending to soften....	Gummata
23	♀	23	Optic neuritis.....	Lues
24	♀	33	Perforation, hard palate.....	Lues
25	♀	28	Lesion on corner of mouth, 1 year old (provocative treatment 1 week ago)	Lues
26	♀	3 mo.	Coryza, loss of weight.....	Lues
27*	♀	55	See 13.....	.....

\* A simple number indicates blood serum. A number with asterisk indicates a duplicate specimen of blood serum from a patient previously tested. Specimens 20\* and 27\* were both from the same patient as 13.

† Specimen 21† was spinal fluid from the same patient who furnished the blood serum Specimen 17.

We come, now, to another group of cases in which again there were no histories of syphilis nor any of antisyphilitic treatment. In some of them the physical findings were rather vague and the clinical diagnoses not stated with any degree of assurance, but in others the findings were quite definitely not those of syphilis and the diagnoses were of conditions not syphilitic, such, for example, as acute articular rheumatism and typhus fever. We shall designate this Group II C. In it there have been placed 16 patients with 16 tests. In all of these there was complete or partial fixation of complement with Antigen B. H. C., either at 37 C. or at 8 C., or both, associated with absence of fixation with either of the other antigens.

The results obtained in this group are summarized in Table 10, and brief data in regard to each test are presented in Table 11.

We feel rather confident that the cases in Group II C were not syphilitic and that the complement-fixation obtained in them was non-



BRIEF DATA CONCERNING 27 TESTS OF 24 PATIENTS EVIDENTLY SYPHILITIC, GROUP II A

[illegible]

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	21	84.0	0	0.0	4	16.0
2. Simple at 37 C. ....	12	48.0	2	8.0	11	44.0
3. Acetone insoluble at 37 C. ....	17	68.0	1	4.0	7	28.0
4. Cholesterinized at 8 C. ....	23	92.0	1	4.0	1	4.0
5. Simple at 8 C. ....	22	88.0	2	8.0	1	4.0
6. Acetone insoluble at 8 C. ....	20	80.0	2	8.0	3	12.0

\* When these results were first compiled, Specimen 1, Table 8, was included in this group. It gave a 2+ reaction with the simple antigen, at 8 C. While this paper was being prepared for publication it was learned that the patient who furnished this specimen of blood was actually a syphilitic. Another specimen from her gave a definite diagnostic Wassermann reaction (Specimen 4, Table 8) and on exploratory laparotomy, the clinical diagnosis of gastric carcinoma was not confirmed.

TABLE 8  
BRIEF DATA CONCERNING 25 TESTS ON 20 PATIENTS PROBABLY SYPHILITIC, GROUP II B

No.	History of Infection	Treatment	Sex	Age	Manifestations at Time of the Tests
1	.....	.....	♀	32	Constipation .....
2	.....	.....	♀	23	Pain in left hip and left side; 2 discharging sinuses in back, right side
3	.....	.....	♀	21	Cutaneous eruption .....
4*	.....	.....	♀	32	Chronic bowel trouble.....
5	.....	.....	♂	26	Nervous; tremulous .....
6	.....	.....	♂	19	Loss of weight; pyloric syndrome.....
7	.....	.....	♂	19	No manifestations .....
8*	.....	.....	♂	19	See 6 .....
9	Doubtful, 1911	.....	♂	26	No manifestations .....
10	.....	.....	♂	64	Acute bronchitis; abdominal pain.....
11	.....	.....	♂	48	Myocarditis; ascites .....
12	.....	None	♂	32	Abdominal pain; epigastric tumor.....
13*	.....	Hg; KI	♂	32	See 12 .....
14	.....	.....	♂	35	Nervousness .....
15	Husband is luetic	.....	♂	22	No manifestations .....
16	.....	.....	♂	48	Ascites; myocarditis .....
17	.....	.....	♂	35	Ascites; jaundice .....
18	.....	.....	♂	42	Hypertrophied turbinate .....
19	.....	.....	♂	10	Mental retardation .....
20*	.....	See 13	♂	32	See 12 and 13.....
21*	.....	.....	♂	35	See 7 .....
22	.....	.....	♂	21	Chronic arthritis, elbow.....
23	.....	.....	♂	58	Headache; facial palsy with anesthesia left side of face; pain in neck
24	.....	.....	♂	36	Acute bronchitis; myocarditis.....
25	.....	.....	♂	29	Epilepsy .....

\* All specimens in this table were blood serums. Specimen 4\* was from the same patient as 1; 8\* from the same patient as 6; 13\* and 20\* from the same patient as 12; 21\* from the same patient as 7.

TABLE 9  
SUMMARIZED RESULTS OF 144 TESTS ON 124 PATIENTS, CERTAINLY OR PROBABLY SYPHILITIC, GROUPS I, II A, AND II B

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	94	65.3	3	2.1	47	32.6
2. Simple at 37 C. ....	54	37.5	4	2.8	86	59.7
3. Acetone insoluble at 37 C. ....	67	46.5	1	0.7	76	52.8
4. Cholesterinized at 8 C. ....	120	83.3	4	2.8	20	13.9
5. Simple at 8 C. ....	106	73.6	6	4.2	32	22.2
6. Acetone insoluble at 8 C. ....	87	60.4	6	4.2	51	35.4

be termed nonsyphilitics. It is unnecessary to show detailed data in these cases. Suffice it to say that there were 341 tests on 317 nonsyphilitics, in none of which was any degree of fixation obtained with any of the antigens, under either condition of incubation.

The tests shown in Table 10 and the results on nonsyphilitics have been combined in Table 12, which shows the results obtained on the

TABLE 8—Continued

BRIEF DATA CONCERNING 25 TESTS ON 20 PATIENTS PROBABLY SYPHILITIC, GROUP II B

Diagnosis	Complement-Fixation							
	At 37 C.				At 8 C.			
	1	2	3	4	1	2	3	4
Carcinoma of stomach	—	—	—	—	—	++	—	—
Tuberculosis or lues	++++	—	—	—	++++	+	—	—
Dermatitis	++++	—	—	—	++++	++++	++	—
Carcinoma of stomach	++++	—	+	—	++++	++++	++	—
.....	++++	++++	++++	—	++++	++++	++++	—
Duodenal ulcer	++++	—	—	—	++++	+	++++	—
.....	—	—	—	—	+	+	+	—
.....	++++	+	++	—	++++	++++	++++	—
.....	++++	+	++++	—	++++	++++	++++	—
Tumor of pylorus	++++	++++	++++	—	++++	++++	++++	—
See 12	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
.....	++++	—	++	—	++++	++++	+	—
.....	++++	++++	++++	—	++++	++++	++++	—
Syphilis	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
See 13	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
Tuberculosis (?); lues (?)	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
.....	++++	++++	++++	—	++++	++++	++++	—
.....	++++	—	—	—	++++	++++	++++	—

TABLE 10

SUMMARIZED RESULTS OF 16 TESTS ON PATIENTS, PROBABLY NOT SYPHILITIC, GROUP II C

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	8	50.0	3	18.8	5	31.2
2. Simple at 37 C. ....	0	0.0	0	0.0	16	100.0
3. Acetone insoluble at 37 C. ....	0	0.0	0	0.0	16	100.0
4. Cholesterinized at 8 C. ....	5	31.2	3	18.8	8	50.0
5. Simple at 8 C. ....	0	0.0	0	0.0	16	100.0
6. Acetone insoluble at 8 C. ....	0	0.0	0	0.0	16	100.0

357 specimens which we consider to be nonsyphilitic. One might obtain an exaggerated idea of the unreliability of the cholesterinized antigen if he regarded only the percentages shown in Table 10. From Table 12 it would appear that this antigen has given a positive result in 2.2% of tests on nonsyphilitic specimens, at 37 C., and in 1.4% of tests on such specimens at the lower incubation temperature, in this series.

TABLE 11

BRIEF DATA CONCERNING 16 TESTS ON 16 PATIENTS, PROBABLY NOT SYPHILITIC, GROUP II C

No.	History of Infection	Sex	Age	Manifestations at Time of the Tests	Diagnosis
1	.....	♂	30	Enlarged liver; jaundice.....	Hepatic cirrhosis
2	.....	♀	22	Acute arthritis.....	Acute articular rheumatism
3	.....	♂	40	Enlarged liver; ascites, jaundice.....	Hepatic cirrhosis
4	.....	♀	26	Pain in lumbar region; loss of weight..	.....
5	.....	♀	20	.....	Hysteria
6	.....	♀	6	.....	Cretin
7	.....	♀	24	Fever, headache, backache, eruption...	Typhus fever
8	.....	♀	37	Abdominal pain.....	Duodenal ulcer
9	.....	♀	25	Nervousness.....	Endocervicitis
10	.....	♀	3	Mental deficiency.....	.....
11	.....	♀	23	Jaundice.....	Catarrhal cholangitis
12	.....	♀	8½	.....	Miliary tuberculosis
13	Father said to be luetic	♀	2½	.....	Idiocy
14	Two babies died under 5 days	♀	25	.....	.....
15	.....	♂	20	Abscess, prepatellar bursa.....	Secondary to wound of leg
16	.....	♀	30	Cervical adenitis.....	Tuberculosis (?); lues (?)

TABLE 12

SUMMARIZED RESULTS OF 357 TESTS ON 333 NONSYPHILITIC PATIENTS, GROUPS II C AND III

Antigen and Temperature	Positive		Doubtful		Negative	
	Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
1. Cholesterinized at 37 C. ....	8	2.2	3	0.8	346	97.0
2. Simple at 37 C. ....	0	0.0	0	0.0	357	100.0
3. Acetone insoluble at 37 C. ....	0	0.0	0	0.0	357	100.0
4. Cholesterinized at 8 C. ....	5	1.4	3	0.8	349	97.8
5. Simple at 8 C. ....	0	0.0	0	0.0	357	100.0
6. Acetone insoluble at 8 C. ....	0	0.0	0	0.0	357	100.0

## SUMMARY

Wassermann tests have been performed on 501 identical specimens from 457 cases by 6 different methods. The cases have been divided into 2 classes, on the basis of the histories, those giving histories of syphilis and those not giving such histories. The 1st class was called Group I. The 2nd class was subdivided into several groups by a consideration of the symptoms and signs, including the Wassermann reaction. Groups II A and II B are composed of cases which presented symptoms and signs of syphilis, more definite in II A than in II B. Both of these subgroups are considered as probably luetic. Group II C comprises cases in which the only evidence of syphilis was

TABLE 11—*Continued*

BRIEF DATA CONCERNING 16 TESTS ON 16 PATIENTS, PROBABLY NOT SYPHILITIC, GROUP II C

Complement-Fixation							
At 37 C.				At 8 C.			
1	2	3	4	1	2	3	4
++	—	—	—	++	—	—	—
++	—	—	—	+	—	—	—
—	—	—	—	++++	—	—	—
—	—	—	—	+	—	—	—
—	—	—	—	+	—	—	—
+	—	—	—	—	—	—	—
++++	—	—	—	—	—	—	—
++++	—	—	—	—	—	—	—
++	—	—	—	—	—	—	—
+	—	—	—	—	—	—	—
++++	—	—	—	—	—	—	—
+	—	—	—	—	—	—	—
++++	—	—	—	++++	—	—	—
—	—	—	—	++++	—	—	—
++++	—	—	—	—	—	—	—
—	—	—	—	++++	—	—	—

complement-fixation with 1 antigen. These cases are considered as probably not syphilitic. Group III is made up of the rest of the cases, in which there was no evidence of syphilis.

The 6 methods consisted in the use of 3 different antigens, namely, alcoholic extract of beef heart, cholesterinized (Antigen B. H. C.), plain alcoholic extract of beef heart (Antigen B. H. P.) and acetone insoluble lipid antigen prepared from the same extract of beef heart (Antigen B. H. A.), under 2 sets of incubation conditions, namely, at 37 C., for 1 hour, and at 8 C., for 4 hours.

It was found that in cases of syphilis and in cases probably syphilitic, the highest percentage of positive reactions was obtained in Method 4, that is, with cholesterinized antigen (B. H. C.), at 8 C., for 4 hours. The other methods, in order of value, were plain antigen (B. H. P.), at 8 C., cholesterinized antigen (B. H. C.), at 37 C., acetone insoluble antigen (B. H. A.), at 8 C., B. H. A., at 37 C., and B. H. P. at 37 C.

Complement-fixation was obtained in a small number of cases which were probably not syphilitic. In all of these cases, the antigen which yielded the fixation was Antigen B. H. C., sometimes at 37 C., sometimes at 8 C., and in a few instances both at 37 C. and at 8 C.



A comparatively large number of negative controls were tested, in none of which, with the exception of the few cited above, which have been considered in a separate group, was any complement-fixation obtained.

#### CONCLUSIONS

1. The use of the cholesterinized antigen, with the first incubation, at 8 C., for 4 hours, constitutes a more sensitive test for syphilis than does any of the other methods examined.

2. The cholesterinized antigen, both at 37 C. and at 8 C., is apt to yield nonspecific complement-fixation. Therefore, in a diagnostic reaction, fixation with the cholesterinized antigen alone is, at best, of only doubtful significance.

3. The simple extract antigen, with the first incubation at 8 C., is more sensitive than the cholesterinized antigen at 37 C., and in this series it did not give any false positive reactions, according to the available evidence.

4. The acetone insoluble preparation, made according to the method of Noguchi, is less sensitive, either at 37 C., or at 8 C., than is the cholesterin-reinforced antigen at either temperature, and is also less sensitive than the simple extract at 8 C. It is more sensitive than the simple extract at 37 C. and, in this series, has, according to the available evidence, given no false positive reactions.

## THERMAL COAGULATION POINT OF BLOOD AND SERUM \*

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It has been definitely demonstrated that certain infectious diseases can be transmitted by a filtrable virus. That is, the infectious agent of these diseases is so small that it can pass through a Berkefeld filter. Among the diseases of this type are poliomyelitis, hog cholera, foot and mouth disease, and mosaic disease of tobacco.

Since the Berkefeld filter will not remove the virus of certain infectious diseases from serum, it becomes necessary to obtain some other means of sterilization. The simplest as well as the most efficient means at our disposal is heat. If we can apply sufficient heat to a product without changing its composition, we have an ideal method of sterilization.

Fortunately, many of the filtrable viruses are destroyed at a comparatively low temperature, that is, at a temperature below the coagulation point of serum. It is possible, therefore, to heat serums to a point sufficient to destroy many of the filtrable viruses without destroying the antibodies or potency of the serums.

The question directly leading up to this discussion is the possibility of the spread of foot and mouth disease by means of hog cholera serum. At least 1 outbreak of foot and mouth disease has been attributed to infected hog cholera serum. It has been found that hog cholera serum can be heated sufficiently to destroy foot and mouth disease virus, which might be present, without apparently changing the physical properties or the potency of the serum. It is important in heating serums to know at what point coagulation will occur, so that this temperature may not be too nearly approximated or the danger point reached.

With this end in view, 67 samples of blood and serums from different animals were heated at varying temperatures, to determine the lowest coagulation point. The heating was carried on in test tubes in a water bath. Each sample was heated for 1 hour. The hog blood

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freshly drawn was defibrinated, and the whole blood heated without the addition of a preservative. A portion of the defibrinated blood was centrifuged and the clear serum was heated without the addition of phenol or tricresol. To another part of the defibrinated blood 1% extract of the navy bean was added to agglutinate the red corpuscles.

TABLE 1  
RESULTS OF THE HEATING OF HOG BLOOD AND HOG SERUM

	56 C.	57 C.	58 C.	59 C.	60 C.	61 C.	62 C.	63 C.	64 C.	65 C.
Hog Blood Defibrinated										
1	—	—	+	+	++					
2	—	—	+	+	++					
3	—	—	—	+	++					
4	—	—	—	+	++					
5	—	—	+	++						
6	—	—	+	+	++					
Hog Serum, without Preservative										
7	—	—	—	—	—	—	—	—	+	++
8	—	—	—	—	—	—	—	+	++	
9	—	—	—	—	—	—	—	—	++	
10	—	—	—	—	—	—	—	—	+	++
11	—	—	—	—	—	—	—	—	+	++
12	—	—	—	—	—	—	—	+	++	
13	—	—	—	—	—	—	—	+	++	
14	—	—	—	—	—	—	—	+	++	
Hog Serum with 1% Bean Extract										
15	—	—	—	—	—	—	—	—	++	
16	—	—	—	—	—	—	—	—	++	
17	—	—	—	—	—	—	—	+	++	
18	—	—	—	—	—	—	—	+	++	
19	—	—	—	—	—	—	—	—	+	++
20	—	—	—	—	—	—	—	—	++	
21	—	—	—	—	—	—	—	—	+	++
22	—	—	—	—	—	—	—	—	—	++
23	—	—	—	—	—	—	—	—	+	++
24	—	—	—	—	—	—	—	+	++	
25	—	—	—	—	—	—	—	—	+	++
26	—	—	—	—	—	—	—	—	—	++

— means no change; + means viscid, increased surface tension; ++ means complete coagulation.

This was then centrifuged in the usual way and the clear serum heated. Table 1 gives the results of the heating of hog blood and hog serum.

It is to be seen from Table 1 that defibrinated hog blood without preservative became viscid in all 6 samples at 58 C., that 1 sample coagulated at 59 C., and that 5 samples coagulated at 60 C.

Hog serum, without preservative, became viscid in 4 samples at 63 C., was coagulated in 5 samples at 64 C., and in 3 samples at 65 C.

Hog serum with 1% bean extract became viscid in 3 samples at 63 C., was coagulated in 6 samples at 64 C., and in 6 samples at 65 C.

In addition to the heating as is shown in Table 1, 12 samples of defibrinated hog blood were heated for 10 hours at 50 C. The blood turned darker in color, but there was no coagulation nor apparent change otherwise.

In order to determine the effect of phenol on the coagulation point, 0.5% phenol was added to 3 samples of defibrinated hog blood, and to 4 samples of hog serum. The defibrinated phenolized blood coagulated at 56 C., and the phenolized serum, at 60 C.

Serum and antitoxin for human and veterinary use are produced from various animals. If we depend entirely on the filtration of a serum for sterility, there is a bare possibility of transmitting some filtrable virus disease through a supposedly harmless serum. This is especially true in the case of transfusion and in the use of human serum. If the filtration process is supplemented by heating the serum, a safer product is obtained.

In order to determine how much heat might be safely applied to serums from various animals, it was decided to test several samples from each of the most commonly used serums.

Serums from horse, sheep, calf, rabbit, guinea-pig, and man were heated to determine the coagulation point. The serum from each animal was prepared by defibrinating the blood mechanically and by centrifuging to remove the corpuscles. The clear serum was used without the addition of any preservative. Each sample was heated for 1 hour in a water bath. Table 2 shows the results of this heating.

Six samples of horse serum were heated; 5 coagulated at 63 C., 1, at 64 C.

All 6 samples of sheep serum coagulated at 65 C. This was to be expected, since all 6 samples were taken from the same sheep at different times. The different samples from the other animals were taken from separate animals.

Calf serum coagulated in 3 samples at 66 C., in 3 others, at 67 C.

Rabbit serum coagulated in 1 sample at 69 C., in 3 samples at 70 C.; 1 sample was viscid but not coagulated at 70 C.

The coagulation point of guinea-pig serum varied from 65 C. to 67 C. Three samples coagulated at 65 C., 4 at 66 C., and 1 at 67 C.

TABLE 2  
RESULTS OF THE HEATING OF HORSE, SHEEP, CALF, RABBIT, GUINEA-PIG AND HUMAN

	60 C.	61 C.	62 C.	63 C.	64 C.	65 C.	66 C.	67 C.	68 C.	69 C.	70 C.
Horse Serum											
27	—	—	—	++							
28	—	—	—	++							
29	—	—	—	++							
30	—	—	—	++							
31	—	—	—	++							
32	—	—	—	+	++						
Sheep Serum											
33	—	—	—	—	—	++					
34	—	—	—	—	+	++					
35	—	—	—	—	+	++					
36	—	—	—	—	—	++					
37	—	—	—	—	—	++					
38	—	—	—	—	+	++					
Calf Serum											
39	—	—	—	—	—	—	++				
40	—	—	—	—	—	—	+	++			
41	—	—	—	—	—	—	+	++			
42	—	—	—	—	—	—	++				
43	—	—	—	—	—	—	—	++			
44	—	—	—	—	—	—	++				
Rabbit Serum											
45	—	—	—	—	—	—	—	—	—	++	
46	—	—	—	—	—	—	—	—	—	—	++
47	—	—	—	—	—	—	—	—	—	—	++
48	—	—	—	—	—	—	—	—	—	+	—
49	—	—	—	—	—	—	—	—	—	—	+
Guinea-pig Serum											
50	—	—	—	—	—	—	++				
51	—	—	—	—	—	—	+	++			
52	—	—	—	—	—	++					
53	—	—	—	—	—	—	++				
54	—	—	—	—	—	++					
55	—	—	—	—	—	++					
56	—	—	—	—	—	+	++				
57	—	—	—	—	—	—	++				
Human Serum											
58	—	—	—	—	++						
59	—	—	—	—	++						
60	—	—	—	—	+	++					
61	—	—	—	++							
62	—	—	—	—	++						
63	—	—	—	—	++						
64	—	—	—	—	++						
65	—	—	—	—	++						
66	—	—	—	—	+	++					
67	—	—	—	—	—	++					

— means no change; + means viscid, increased surface tension; ++ means complete coagulation.



Human serum showed some slight variation. One sample coagulated at 63 C., 6 samples at 64 C., and 3 samples at 65 C.

## SUMMARY

Defibrinated hog blood was heated for 10 hours at 50 C., without coagulation.

Defibrinated hog blood became viscid at 58 C., and coagulated at 60 C.

Defibrinated hog blood with 0.5% phenol coagulated at 56 C.

Hog serum, without preservative, became viscid at 63 C., coagulated at 64 and 65 C.

The addition of 1% bean extract to hog serum did not affect the coagulation point.

The addition of 0.5% phenol to blood or serum gave a marked reduction in the coagulation point.

Horse serum coagulated at 63 and 64 C.

Sheep serum coagulated at 65 C.

Calf serum coagulated at 66 and 67 C.

Rabbit serum coagulated at 69 and 70 C.

Guinea-pig serum coagulated at 65, 66, and 67 C.

Human serum coagulated at 63, 64, and 65 C.

# COMPARISON BETWEEN THE SUBCUTANEOUS AND INTRACUTANEOUS METHODS OF TESTING THE VIRULENCE OF DIPH- THERIA BACILLI \*

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For final diagnosis no method exists, other than that of animal inoculation, for differentiating between virulent diphtheria bacilli and nonvirulent diphtheria-like organisms. In diagnostic laboratories this fact is of economic importance on account of the number of guinea-pigs that must be used. The method usually employed is to isolate the organism, grow it for 48 hours in ascitic broth, and inject 1-2 c.c. of the culture subcutaneously into a guinea-pig. A control pig is injected with the same amount of the broth culture and also a protecting amount of antitoxin. If the organism is the diphtheria bacillus, the test pig will die within 2-3 days and the control pig will live. On necropsy, the test pig will show the typical lesions, that is, subcutaneous infiltration at the site of injection and hemorrhagic suprarenals. On the whole, this method gives satisfactory results, but it necessitates the use of 2 pigs for each test.

Recently, Zingher<sup>1</sup> has employed a method (suggested by the Neisser intracutaneous test) whereby 2 pigs may be used for 4-6 tests: the total time necessary to carry through a test being 4-6 days, as compared to 5-7 days for the subcutaneous method. This method, therefore, effects a saving both in time and animals.

Neisser recommended that the virulence of cultures be tested in the following way:

One loopful of a 24-hour Loeffler slant of the organism is suspended in 1 c.c., 10 c.c., and 100 c.c. of physiologic sodium chlorid solution, and 0.1 c.c. of each suspension is injected intracutaneously on the abdominal surface of a guinea-pig. True diphtheria bacilli will incite a local inflammatory lesion with superficial necrosis in 48-72 hours, the intensity of the reaction depending on the number of injected organisms and their virulence. As a control, antitoxin containing 8 units per c.c., is added to an equal volume of the heaviest sus-

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<sup>1</sup> Proc. New York Path. Soc., 1915, 15, p. 18.

pension, and 0.1 c.c. of the mixture is injected intracutaneously into the same guinea-pig. The skin at the site of the control injection should remain normal in appearance.

Zingher found the use of 1 pig only for the test and control unsatisfactory, in that the direct addition of antitoxin in the control injection so immunized the animals that the test lesions were affected to a considerable degree. Further, when the amount of antitoxin was diminished to avoid the general immunization, the local action of the bacteria was not completely inhibited and lesions were found in both test and control areas. Zingher modified the technic as follows:

Two guinea-pigs are used for testing 4-6 strains. The hair on the abdominal surface is removed and 4 or 6 areas are marked according to the size of the pig. One pig serves as a control and receives about 100 units of antitoxin intracardially at the time of making the tests, or intraperitoneally 24 hours before. A bacterial suspension is made from a fresh 24-hour growth from an ordinary Loeffler's slant suspended in 20 c.c. of physiologic sodium chlorid solution; 0.15 of this emulsion is injected intracutaneously into both pigs. A very fine steel or platinum iridium needle should be used. If the injection is properly made, a circumscribed elevation appears which persists 1 or 2 minutes. Virulent strains induce a definite local inflammatory lesion which shows a superficial necrosis in 48-72 hours. In the control pig the skin remains normal. With nonvirulent strains no lesion will be found in either control or test animals.

In order to compare the value of the 2 methods of testing the virulence of diphtheria bacilli, 37 organisms were isolated from the throats of positive or suspected cases.

For the subcutaneous tests, the organisms were grown in meat infusion broth (neutral to phenolphthalein) to which 10% horse serum was added. After an incubation period of 48 hours, 2 c.c. of this toxin culture were injected into a test pig. A control pig received the same amount of culture and at the same time 100 units of antitoxin. Zingher's modification was used for the intracutaneous tests.

Twenty-two of the 37 cultures gave identical results by both methods. Fourteen of these were virulent and 8 were nonvirulent. In the virulent cases, the test guinea-pigs which were injected intracutaneously developed necrosis at the site of inoculation, while the control pigs showed no reaction. Those injected with toxin subcutaneously died within 3 days and the corresponding control pigs remained unaffected.

At this time a number of animals were lost because of an infectious disease that spread among them. This necessitated the repetition of 15 tests. A possibility of error in the subcutaneous method was

TABLE 1  
RESULTS OF TESTS OF 37 CULTURES BY INTRACUTANEOUS AND SUBCUTANEOUS METHODS

No.	Virulent Strains		No.	Nonvirulent Strains	
	Intracutaneous Method	Subcutaneous Method		Intracutaneous Method	Subcutaneous Method
1	Inflammatory infiltration.....	Test pig died	1	No necrosis.....	Test pig did not die
2	Necrosis.....	Test pig died	2	No necrosis.....	Test pig did not die
3	Necrosis.....	Test pig died	3	No necrosis.....	Test pig did not die
4	Necrosis.....	Test pig died	4	No necrosis.....	Test pig did not die
5	Necrosis.....	Test pig died	5	No necrosis.....	Test pig did not die
6	Necrosis.....	Test pig died	6	No necrosis.....	Test pig did not die
7	Necrosis.....	Test pig died	7	No necrosis.....	Test pig did not die
8	Necrosis.....	Test pig died	8	No necrosis.....	Test pig did not die
9	Necrosis.....	Test pig died	9	No necrosis.....	Test pig did not die
10	Necrosis.....	Test pig died	10	No necrosis.....	Test pig did not die
11	Necrosis.....	Test pig died	11	No necrosis.....	Test pig did not die
12	Slight necrosis.....	Test pig died	12	No necrosis.....	Test pig died†
13	Slight necrosis.....	Test pig died	*	No necrosis.....	Test pig did not die
14	Necrosis.....	Test pig died	13	No necrosis.....	Test pig died†
15	Necrosis.....	Test pig died	*	.....	Test pig did not die
16	Necrosis.....	Test pig died	14	No necrosis.....	Test pig died†
17	Necrosis.....	Test pig died	*	No necrosis.....	Test pig did not die
18	Necrosis.....	Test pig died			
19	Necrosis.....	Test pig died			
20	Necrosis.....	Test pig died			
21	Necrosis.....	Test pig did not die			
*	Necrosis.....	Test pig died			
22	Necrosis.....	Test pig did not die			
*	Necrosis.....	Test pig died			
23	No necrosis.....	Test pig died			
*	Necrosis.....	Test pig died			

\* Second test.

† Death due to an infectious disease.

brought to light as a result. In 3 cases, the test pigs died while the control pigs lived. This would undoubtedly have led to the conclusion that the control pigs were protected by the antitoxin received, and that the organism injected was the true diphtheria bacillus, had not the test pigs injected intracutaneously showed no lesions.

Necropsy findings in pigs dying from diphtheria were not always constant. The suprarenal bodies showed varying degrees of congestion. In 1 case the diphtheria bacillus was isolated from the heart's blood but the suprarenal bodies were apparently normal.

Variations in the susceptibility of the different animals used appeared to influence the results in a small degree. In 2 instances the test pigs inoculated subcutaneously did not react, while the test pigs inoculated intracutaneously showed marked necrosis. A repetition of the tests gave conclusive evidence that the organisms injected were virulent and that the failure to produce death was in all probability due to a certain amount of natural antitoxin possessed by the individual guinea-pigs.

In the other instance the 1st test pig injected intracutaneously was apparently insusceptible.

From the number of cultures tested, the intracutaneous appears to give more accurate results than the subcutaneous method and has a further advantage of being more economical in time and animals. A possible disadvantage lies in the fact that if 1 toxin pig dies, 4 or 6 tests must be repeated.



## EXPERIMENTS IN FILTRATION OF ANTIHOG- CHOLERA SERUM \*

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In accordance with the suggestions of Dorset and Henley<sup>1</sup> on the production of clear and sterilized antihog-cholera serum, we have attempted to produce bacteria-free serum by passage through Berkefeld V and Chamberland F. filters. The results of experiments in this connection have led to other investigations which are presented in this article.

The Chamberland F. candles had been employed in former filtrations and were burned free of organic matter before being used in these experiments. New Berkefeld V candles were used in each instance. In conducting the filtrations no accurate data were recorded of the temperature, duration, or pressure exercised in the process of filtration. No dilutions were made of the material used for filtration. Sterility of all filtrates was determined by plating with ordinary agar-agar mediums and subsequent incubation for 48 hours at 37.5 C.

The whole serum used represents the defibrinated, phenolized blood of hyperimmunized hogs, as obtained in the routine method of antihog-cholera-serum production employed at these laboratories. The virus is the defibrinated, phenolized blood of cholera-sick hogs, and is the strain of virus regularly used at this institute in serum testing. All products used in these experiments were retained in storage at a temperature of 48-52 F.

For testing the potency of the various substances, individual litters of pigs were employed. The animals weighed 30-60 pounds and were fair representatives of pigs generally used for testing antihog-cholera serum. The pigs received 2 c.c. each of virus. All animal inoculations were made intramuscularly.

An experiment was made with blood obtained from 2 hyperimmunized hogs.

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<sup>1</sup> Jour. Agric. Research, 6, p. 333.

A portion of the defibrinated blood was carbolized by adding 0.5% of carbolic acid crystals from a 5% dilution of the acid and placed in storage. Another portion was prepared according to the method of Dorset and Henley:

To each 100 c.c. of the cool defibrinated blood add 1 c.c. of the sterile bean extract and stir to secure a uniform mixture. Allow the mixture to stand until agglutination is clearly evident. This can be determined by examining a small amount in a glass or tube. Agglutination is usually apparent within five minutes after adding the bean extract. There should then be added 1 gm. of finely powdered sodium chlorid. The salt is stirred in until dissolved, and the mixture of defibrinated blood, bean extract and salt is allowed to stand for about 15 minutes.

The agglutinated blood was centrifuged and the supernatant serum poured off and passed through a Chamberland F. filter. The whole serum and Chamberland filtrate were tested for comparative potency on a litter of 7 pigs. The test was started Oct. 5, 1916.

TABLE 1  
RESULTS OF TEST 1, USING CHAMBERLAND FILTRATE; AMOUNT OF VIRUS, 2 C.C.

Date, October	Number and Weight of Pig						
	34 41 lbs.	35 35 lbs.	36 42 lbs.	37 40 lbs.	38 50 lbs.	39 49 lbs.	40 51 lbs.
	Virus Con- trol	Whole Serum			Chamberland Filtrate		
		5 C.c.	10 C.c.	15 C.c.	5 C.c.	10 C.c.	15 C.c.
5	101.2	101.6	102.0	101.0	101.4	101.0	101.2
6	100.0	102.0	102.8	102.0	100.2	101.4	101.2
7	100.2	100.4	101.0	101.2	99.4	99.0	99.6
8	102.4	101.2	102.6	101.8	100.8	101.6	101.0
9	104.0	102.0	103.0	102.4	103.0	102.4	102.8
10	104.8	101.8	102.2	101.4	102.0	100.6	102.8
11	105.0	102.0	102.4	101.6	104.0	101.8	103.4
12	105.8	103.0	104.8	103.2	104.4	102.8	103.8*
13	106.0*	102.8	104.6	102.0	105.8	105.4	105.8
14	106.4d	101.8	102.4	101.6	106.2*	105.8	106.6*
15	106.8*	101.0	101.6	101.4	106.4*	104.8*	106.0*
16	106.4*	101.0	101.2	101.4	107.0*d	106.6*d	107.2*d
17		100.4	100.2	101.0	105.8*d	106.2*d	106.0*d
18		100.2	101.0	101.4		105.8*d	
19		101.4	101.0	101.2			
20		101.4	101.4	102.4			
21		101.2	101.0	101.2			
22		101.0	101.2	101.4			
23		100.2	101.0	100.4			
24		100.0	100.2	101.4			
25		100.2	101.0	98.6			
	Died; acute hog cholera	Remained well	Remained well	Remained well	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera

\* Loss of appetite; d, diarrhea.

TEST 1.—The record of Test 1 shows that the virus control pig died 11 days after inoculation and showed lesions of acute hog cholera. The pigs receiving 5, 10, and 15 c.c. of whole serum respectively, remained well throughout the test. The pigs receiving 5, 10, and 15 c.c. of Chamberland filtrate respectively, died on the 12th and 13th days after injection. Each of these pigs presented lesions of hog cholera.

From this test it is evident that the whole serum protected, while the Chamberland filtrate from the same source would not protect in

the amounts employed. The centrifuged serum was not tested in this instance as it was assumed from experiments by Dorset and Henley that clear, centrifuged serum is equally as potent as the whole serum. A duplicate test was made, with material from the same source, on a litter of 10 pigs.

TEST 2.—The virus control pig died 8 days after inoculation and showed lesions of acute hog cholera. The pigs received 5, 10, 15, and 20 c.c. of Chamberland filtrate, respectively, died within 12 days following inoculations and showed symptoms and postmortem lesions of hog cholera. The pig receiving

TABLE 2  
RESULTS OF TEST 2, USING CHAMBERLAND FILTRATE; AMOUNT OF VIRUS, 2 C.C.

Date, October, November	Number and Weight of Pig									
	41 35 lbs.	42 45 lbs.	43 49 lbs.	44 45 lbs.	45 47 lbs.	46 56 lbs.	47 52 lbs.	48 55 lbs.	49 55 lbs.	50 59 lbs.
	Virus Control	Chamberland Filtrate				Whole Serum				Serum Control
		5 C.c.	10 C.c.	15 C.c.	20 C.c.	5 C.c.	10 C.c.	15 C.c.	20 C.c.	20 C.c.
12	102.4	102.0	102.8	103.2	101.8	101.4	102.2	102.2	102.8	101.2
13	101.2	101.8	102.0	102.2	102.4	101.2	102.6	102.2	102.4	102.0
14	102.4	101.4	102.0	102.0	102.6	102.0	101.4	102.0	101.6	102.2
15	104.2	104.4	105.0	102.4	104.2	102.0	101.4	101.8	104.2	101.6
16	105.2	105.2	106.0	103.8	104.4	102.8	104.6	102.4	104.0	103.8
17	106.2	105.4	106.0	105.2	105.0	104.4	104.4	103.6	104.6	104.0
18	106.0d	104.8	105.8d	105.8	104.0	105.4	103.8	103.2	104.0	103.6
19	104.6*d	106.2*d	106.4*d	106.4*d	105.8*d	105.8	105.4	104.2	102.0	104.6
20	101.8*d	105.8*d	105.4*d	106.4*d	106.6*d	105.4*d	103.6	102.4	102.4	102.2
21		105.0*d	100.2*d	106.0*d	104.2*d	104.6d	102.0	101.8	101.6	101.4
22		106.0*d		107.4*d	104.2*d	105.2*d	101.8	102.0	101.2	101.6
23		105.4*d		106.4*d	105.8*d	104.8*d	101.6	100.4	100.6	102.2
24		106.6*d			107.2*d	107.2*d	101.8	100.2	101.0	101.2
25						106.4*d	102.0	101.4	101.0	102.6
26							102.6	101.0	99.8	101.6
27							101.4	101.0	100.6	104.8
28							101.2	101.6	101.2	102.4
29							102.0	101.6	100.4	100.6
30							101.0	100.2	101.0	100.4
31							100.4	100.4	100.0	100.2
1							100.4	100.2	101.2	101.4
2							101.0	100.2	101.2	101.4
	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera	Re- mained well	Re- mained well	Re- mained well	Re- mained well

\* Loss of appetite; d, diarrhea.

5 c.c. of whole serum, died 13 days after injection and showed lesions of hog cholera. The pigs receiving 10, 15, and 20 c.c. of whole serum, respectively, remained healthy. The serum control, receiving 20 c.c. of a tested serum, remained well during the test.

The results obtained from the 1st 2 tests with Chamberland filtrate and whole serum led us to experiments using Berkefeld candles in order to determine if these candles also would hold back immune bodies, and further if the residuum would contain a concentration of these bodies. The Berkefeld filtrate thus obtained was in turn passed

through a Chamberland F. filter in order to determine whether or not there were immune bodies present in the filtrate which could be restrained by a candle of this nature.

Blood for this experiment was obtained from 11 hyperimmunized hogs. One portion was agglutinated, centrifuged, and carbolized, as in the former test; 800 c.c. of the centrifuged serum were used for Berkefeld filtration and 500 c.c. of filtrate and 200 c.c. of residuum

TABLE 3  
RESULTS OF TEST 3 A, USING BERKEFELD AND CHAMBERLAND RESIDUUM AND FILTRATE;  
AMOUNT OF VIRUS, 2 C.C.

Date, January, February	Number and Weight of Pig						
	233 51 lbs.	234 46 lbs.	235 55 lbs.	236 55 lbs.	237 41 lbs.	238 47 lbs.	239 55 lbs.
	Virus Control.	Whole Serum, 10 C.c.	Centri- fuged Serum, 10 C.c.	Berke- feld Residuum, 10 C.c.	Berke- feld Filtrate, 10 C.c.	Chamber- land Residuum, 10 C.c.	Chamber- land Filtrate, 10 C.c.
10	101.4	102.0	101.8	101.8	101.6	101.8	101.6
11	100.2	102.4	102.0	103.8	101.4	102.0	102.6
12	101.4	101.4	101.0	101.2	101.8	102.6	102.0
13	103.0	102.8	103.0	101.4	101.2	103.0	102.4
14	105.4	101.6	102.8	102.0	104.4	104.2	104.0
15	104.6	102.2	104.6	101.8	104.6	104.0	104.0
16	104.2	102.4	104.0	101.0	105.2	104.6	104.0
17	104.0	101.6	103.0	101.6	106.0*	103.4	104.2
18	105.2	102.0	104.0	101.8	106.4*	105.4	105.8
19	106.2*	101.2	102.6	100.2		107.0*	105.4*
20	104.6	101.8	103.4	100.4		106.1*	105.8*
21	105.0	102.2	104.0	101.6		106.6*	105.0*d
22	106.0*	100.6	104.4	102.6		106.4*	105.2*d
23	106.4	101.0	103.4	101.4		107.0*	
24	106.4	102.4	103.2	101.8		106.6*	
25	104.4*	103.0	103.2	101.2		107.4*d	
26	105.2*	103.4	103.0	101.2		104.8*d	
27	105.4*	101.4	102.6	101.8		101.6*d	
28	105.0*	101.2	102.8	101.4			
29	103.0*d	101.0	102.6	101.8			
30		101.4	102.2	101.2			
31		101.8	101.4	102.6			
1		101.8	101.8	101.4			
	Died; acute hog cholera	Remained well	Remained well	Remained well	Died; acute hog cholera	Died; acute hog cholera	Died; acute hog cholera

\* Loss of appetite; d, diarrhea.

were secured; 400 c.c. of the filtrate were used for passage through a Chamberland F. candle, and from this were obtained about 125 c.c. of Chamberland filtrate and about 250 c.c. residuum. The amounts of the various filtrates and residua obtained from these filtrations are only approximate, as no special effort was made to collect the largest amounts available.

In this experiment whole serum, centrifuged serum, Berkefeld residuum, Berkefeld filtrate, Chamberland residuum and Chamberland

filtrate were tested for comparative potency. Two litters of pigs, 7 in each, were used to determine the potency of these various products. A dosage of 10 c.c. each was used for injecting the pigs of 1 litter, and a dosage of 20 c.c. each was employed in pigs of the other litter. This test was started Jan. 10, 1917.

TEST 3A.—In the litter of pigs receiving the smaller dosage, the virus control pig died 19 days after injection with lesions of acute hog cholera. The pigs receiving 10 c.c. of whole serum, centrifuged serum, and Berkefeld

TABLE 4  
RESULTS OF TEST 3 B, USING BERKEFELD AND CHAMBERLAND RESIDUUM AND FILTRATE;  
AMOUNT OF VIRUS, 2 C.C.

Date, January, February	Number and Weight of Pig						
	240 50 lbs.	241 60 lbs.	242 52 lbs.	243 38 lbs.	244 58 lbs.	245 54 lbs.	246 48 lbs.
	Whole Serum, 20 C.c.	Centri- fuged Serum, 20 C.c.	Berke- feld Residuum, 20 C.c.	Berke- feld Filtrate, 20 C.c.	Chamber- land Residuum, 20 C.c.	Chamber- land Filtrate, 20 C.c.	Serum Control, 20 C.c.
10	102.0	101.2	100.6	101.8	101.0	101.2	101.4
11	101.8	103.0	104.0	102.0	102.6	101.0	102.0
12	101.6	102.0	102.2	101.2	103.2	101.0	101.0
13	102.6	102.6	101.8	102.0	103.0	101.8	102.0
14	103.0	103.2	102.4	103.0	103.8	104.0	102.2
15	103.2	103.0	101.2	102.0	104.2	102.8	101.0
16	102.6	101.8	102.0	102.4	104.0	104.2	100.4
17	102.4	101.2	102.4	102.6	104.4	103.0	101.2
18	102.4	101.8	103.0	102.0	104.8	104.2	102.0
19	101.6	100.4	102.0	102.0	104.4	102.8	101.0
20	102.0	100.2	102.0	103.4	105.8*	103.8	100.0
21	102.4	101.0	102.0	104.8	104.8	103.2	101.2
22	101.2	100.2	101.2	105.4	105.0	106.2	100.2
23	101.4	101.6	102.0	105.6*	105.8*	106.4*	100.2
24	101.8	101.4	102.0	106.0*	105.4*	107.8*	101.2
25	102.6	101.8	103.0	105.6*	105.0*		101.2
26	101.6	101.6	102.0	103.0*	104.8*		100.2
27	101.4	101.4	101.6	102.0	105.8*		101.0
28	101.2	100.2	101.6	101.8	103.2*d		100.0
29	102.0	101.6	101.2	102.0			100.2
30	102.2	101.6	101.8	101.6			101.6
31	101.4	101.0	101.2	103.8			101.2
1	102.0	101.4	101.8	103.8			101.0
	Remained well	Remained well	Remained well	Recovered, very sick	Died; acute hog cholera	Died; acute hog cholera	Remained well

\* Loss of appetite; d, diarrhea.

residuum, respectively, remained well during the test. The pigs receiving 10 c.c. of the Berkefeld filtrate, Chamberland residuum, and Chamberland filtrate, respectively, died 8-17 days after injection with lesions of hog cholera on necropsy.

TEST 3B.—In the 2nd litter, the pigs that received 20 c.c. of whole serum, centrifuged serum, and Berkefeld residuum, respectively, remained healthy. One pig, receiving 20 c.c. of Berkefeld filtrate, showed a marked fever and physical reaction, but eventually recovered. The pigs receiving 20 c.c. of Chamberland residuum and Chamberland filtrate, respectively, died on the 14th and 18th day following injection with lesions of hog cholera. Serum control



pig which received 20 c.c. of a tested serum, remained healthy throughout the test.

The results of the experiments would indicate that the Berkefeld as well as the Chamberland filters restrain the immune bodies of antihog-cholera serum.

From Test 3 it is evident that the whole serum, centrifuged serum, and Berkefeld residuum, protected in 10 c.c. doses; while 10 c.c. of Berkefeld filtrate, Chamberland residuum, and Chamberland filtrate failed to protect the test animals against hog-cholera virus.

TABLE 5

RESULTS OF TEST 4, USING BERKEFELD RESIDUUM; AMOUNT OF VIRUS, 2 C.C.

Date, February	Number and Weight of Pig									
	285 32 lbs.	286 32 lbs.	287 33 lbs.	288 30 lbs.	289 33 lbs.	290 32 lbs.	291 32 lbs.	292 30 lbs.	293 31 lbs.	294 36 lbs.
	Virus Control	Whole Serum				Berkefeld Residuum				Serum Control
	2.5 C.c.	5 C.c.	10 C.c.	15 C.c.	2.5 C.c.	5 C.c.	10 C.c.	15 C.c.	15 C.c.	
8	103.6	103.4	103.0	103.0	103.6	103.4	103.0	103.6	103.0	103.2
9	103.0	103.4	103.2	102.6	103.4	103.6	104.4	103.8	104.2	104.0
10	102.0	101.8	102.4	101.8	102.6	101.6	102.8	102.4	101.8	101.0
11	101.8	101.4	101.6	101.6	102.0	100.2	102.0	100.4	101.0	101.2
12	104.4	104.0	103.8	102.6	102.8	104.0	102.6	102.4	102.4	102.6
13	102.0	101.2	101.8	101.6	101.0	102.8	102.6	102.6	102.8	103.0
14	103.6	104.2	101.6	102.6	103.8	102.6	102.4	102.2	102.4	102.4
15	105.0	103.4	102.4	101.8	103.2	101.6	102.2	101.8	101.8	103.4
16	103.2	105.0	103.0	104.0	104.0	101.4	101.8	101.2	102.0	102.4
17	105.6	104.4	105.4	103.6	105.0	102.0	101.8	103.2	102.8	102.0
18	106.4*	107.0*	105.6*	105.4*	104.6	101.8	102.0	102.6	102.0	101.6
19	105.8	106.8*	106.0*	103.8	103.8	103.2	101.0	104.4	102.2	101.0
20	106.2*	105.4 <sup>d</sup>	104.8*	105.0	102.0	103.0	101.2	102.8	101.6	101.8
21	106.6		103.2	103.8	102.6	104.2	101.2	102.8	101.0	101.4
22	107.0 <sup>d</sup>		104.4	105.2	102.8	102.6	101.0	101.8	100.2	102.4
23	106.4* <sup>d</sup>		103.6*	104.6	102.0	101.8	101.6	101.0	101.2	101.0
24	105.4*		103.8*	104.4	102.6	102.0	101.6	101.2	100.4	100.2
25			104.2	104.0	102.8	101.6	101.0	101.8	101.0	101.2
26			104.0*	104.0	105.6	102.4	101.0	102.0	102.2	101.8
27			103.0	102.8	103.8	102.0	100.6	102.0	101.6	101.0
28			102.2	102.0	102.6	101.2	101.2	101.0	101.0	101.1
	Died; acute hog cholera	Died; acute hog cholera	Recov- ered, very sick	Recov- ered, sick	Recov- ered	Re- mained well	Re- mained well	Re- mained well	Re- mained well	Re- mained well

\* Loss of appetite; d, diarrhea.

Since the Berkefeld filtrate, as demonstrated by this test, is deficient in immune bodies, it is reasonable to believe that a concentration of these bodies exists in the Berkefeld residuum. A 4th experiment was made in order to determine the relative potency of whole serum and Berkefeld residuum. A litter of 10 pigs was used for this test, which was started Feb. 8, 1917.

TEST 4.—The virus control died 16 days after the inoculation and showed lesions of acute hog cholera. Of the pigs which received 2.5, 5, 10, and 15 c.c. of whole serum, respectively, the one which received 2.5 c.c. died 12 days

after injection and showed lesions of acute hog cholera; the others recovered. The pigs which received 2.5, 5, 10, and 15 c.c. of Berkefeld residuum, respectively, remained normal. The serum control, received 15 c.c. of a tested serum and remained well. In this test the Berkefeld residuum protected more satisfactorily, in these amounts, than did the whole serum.

#### SUMMARY

The results obtained in the first 2 tests indicate that the immune bodies of antihog-cholera serum, when prepared as stated, are restrained by filtration through Chamberland F. filters.

The 3rd test shows that the Berkefeld as well as the Chamberland filter restrains immune bodies of antihog-cholera serum.

When Berkefeld filtrate was in turn passed through a Chamberland F. filter, the resulting residuum was not sufficiently potent in a dose of 20 c.c. to protect the test animal against simultaneous inoculation with 2 c.c. of hog-cholera virus.

In the 4th test the Berkefeld residuum protected more satisfactorily than did the whole serum.

We were unable to obtain a potent, bacteria-free antihog-cholera serum by filtration through either Berkefeld or Chamberland F. candles.

Blood serum being rich in proteins, adsorption of these according to Bechhold, Rosenthal, and others,<sup>2</sup> always takes place in the filter pores, so that finally the filtration is actually through a colloid filter. With this condition existing, the 1st portion of the filtrates passing through the candles may have contained the immune bodies of antihog-cholera serum, while the latter portion may have been free of these bodies.

While it may be possible to produce a potent, bacteria-free antihog-cholera serum by filtration of serum obtained as the result of normal clotting or by centrifuging defibrinated blood, experiments to determine this were not conducted.

<sup>2</sup> Am. Vet. Review, 1914, 16, p. 134.

# THE SCHICK TEST, WITH ESPECIAL REFERENCE TO THE NEGRO \*

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The Schick test is a simple intracutaneous test that indicates whether or not the person tested possesses a natural antitoxic immunity to diphtheria. Numerous recent observations in this country have not only confirmed the results obtained by Schick,<sup>1</sup> the originator of the test, but have brought out many additional points concerning its value and applications. The practical value of the test in the management and control of diphtheria has been definitely established.

Zingher,<sup>2</sup> in describing the reaction, says: "This reaction is characterized by a circumscribed area of redness and slight infiltration which measures from 1.0-2.5 cm. in diameter. It persists for seven to ten days, and on fading shows superficial scaling and a persistent brownish pigmentation." Practically all other observers describe it in identically the same way, and such is a true description of the reaction as it occurs in white people. All writers also agree that scaling and pigmentation are characteristic of all positive reactions.

This study was made to determine the following 2 points: 1. What color the pigmentation would be on very dark or black skins. 2. The degree of natural immunity to diphtheria that negroes possess, as evidenced by this test. Zingher,<sup>2</sup> Graef and Ginsberg,<sup>3</sup> Neff,<sup>4</sup> and others have carried out the test on negroes, but none of them make any mention of the above stated points.

This article is based on work done during an outbreak of diphtheria among the nurses and patients at the Freedmen's Hospital, Washington, D. C., which began Dec. 20, 1915, and continued throughout the month of January, 1916. Briefly, the test was carried out in the following manner:

Diphtheria toxin 14 months old was used. The toxin was diluted immediately before using with physiologic sodium chlorid solution, so that 0.2 c.c. represented  $\frac{1}{50}$  M. L. D. of toxin for a 300 gm. guinea-pig. I used 0.2 c.c.

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<sup>1</sup> München. med. Wchnschr., 1913, 60, p. 2608.

<sup>2</sup> New York State Jour. Med., 1916, 16, p. 118.

<sup>3</sup> Jour. Am. Med. Assn., 1915, 64, p. 1205.

<sup>4</sup> Ibid., p. 585.

of the diluted toxin for each injection. All injections were made intradermally. The site chosen was the flexor surface of the forearm near the middle. In making the injection I discovered by experience that the method of inserting the needle into the skin, advocated by Rappaport,<sup>5</sup> is very efficacious; and therefore I used it in most of these cases, although I was unaware at the time that anyone else was using it, as Rappaport's article was published subsequent to this work.

The test was carried out on 168 patients, 32 nurses, and 10 interns, making a total of 210 persons tested. Of the 210 persons, 207 were colored, while the remaining 3 were white. The color of the skin of the persons tested ranged from white to black. Light brown, dark brown, and black skins predominated. The ages were between 20 and 40 years; most were between 25 and 30 years. There were 135 females and 75 males in the group. None had received any previous injections of antitoxin.

Of the 210 persons tested, 86, or 40.95%, reacted positively, while 124, or 59.05%, reacted negatively. Six patients, not included in the preceding number, who had, 1 week before, received 1500 units of diphtheria antitoxin, were tested; they all reacted negatively. According to sex, the reactions were as indicated in Table 1.

TABLE 1  
REACTIONS TO TEST, ACCORDING TO SEX

	Test +	Test —	Total
Males.....	26 or 34.2 %	49 or 65.8 %	75
Females.....	60 or 44.44%	75 or 55.56%	135

Pseudoreactions occurred in 10 cases; 6 of these were in persons who gave a positive Schick test, while 4 occurred in negative cases. These pseudoreactions were differentiated from true positive reactions by the following points, as established by Park, Zingher, and Serota,<sup>6</sup> namely: that they appear earlier, are more infiltrated, less sharply circumscribed, disappear in 24-48 hours, and leave only a faintly pigmented area that never shows scaling. Cases showing the combined reaction offer greater chances of errors in reading than do cases that give a negative Schick test. Unfortunately, Zingher's method<sup>7</sup> of control of these reactions had not appeared at the time this work was done, but I believe that its use will materially lessen the mistakes caused by the occurrence of these pseudoreactions.

<sup>5</sup> Jour. Am. Med. Assn., 1916, 66, p. 1448.

<sup>6</sup> Arch. Pediat., 1914, 31, p. 483.

<sup>7</sup> Jour. Am. Med. Assn., 1916, 66, p. 1617.

Itching is the symptom most commonly described. In this series itching occurred rather infrequently, while by far the great majority of these people complained of local soreness. A few complained of slight degrees of pain. One unusually severe reaction occurred, which I am inclined to attribute to a hypersensitive condition of the patient's skin, as a 0.2 c.c. dilution of toxin was used and not a 0.1 c.c. dilution.

Over the zone of reaction in every positive case, there was an exaggeration of the normal lines of the skin together with a definite and, in some cases, marked roughening of the skin. The exaggerated normal skin lines resembled the grayish trails or striae seen on the surface of papules in lichen planus, and to which the term 'lichenification' could very appropriately be applied, just as it is to all other lesions that resemble lichen planus in this respect.

Daily measurements were made of the diameters of the reaction areas in all of the positive cases and records were kept. A maximum diameter was reached in 7 cases at the end of 24 hours, in 38 cases at the end of 48 hours, in 24 cases at the end of 72 hours, in 9 cases at the end of 4 days, in 1 case at the end of 5 days, and in 1 case at the end of 6 days. The 6 cases that showed combined reactions were not included in the preceding number. It is seen from the grouping of 80 cases, that the reaction area reaches its greatest size on the 2nd, 3rd, and 4th days, in most instances. The diameters of the reaction areas varied 1-3 cm.; most measured 1.2-1.4 cm.

Scaling occurred in all undoubtedly positive cases. The scales are often described as being 'silvery white' in color. In all of these cases the scales were white or grayish in color, even in very dark-skinned persons. A slight amount of infiltration and elevation was evident over the zone of reaction on palpation in every positive case.

As regards the pigmentation, it was found that the darker the skin, the darker the pigmentation. In white and very fair skins the pigmentation was of a light brown color, while in darker skins the pigmentation was darker than the color of the skin of the person tested. This was found to be true in every case; in some cases the pigmentation was black in color. This point is of especial interest to me, as the question was raised when I was beginning this work, whether I should be able to make satisfactory readings on very dark or black skins. I found the pigmentative reaction to be equally as clearcut and definite in all the cases I tested. From a scientific standpoint, it is only what one would expect, that is, that a pigmented race should show a greater pigmentative reaction to a pigment-producing stimulus than a non-



pigmented race. Occasionally, one does encounter a negro, who is literally so black that an increase in pigmentation is obviously an impossibility; but such persons are very rarely met with in this country. In such persons the exaggeration of the normal skin lines would be very noticeable and indicative of a positive reaction.

No person reacting negatively to this test received any prophylactic doses of antitoxin and none developed clinical diphtheria.

Two cases seemed to me to be of sufficient interest to warrant describing:

One was a woman who, 48 hours after injection, showed an erythematous area on her arm about 1 cm. in diameter; during the following night she developed a membrane in her throat, which was seen the next morning; at this time the diameter of the reaction area had increased to 1.5 cm.; she was immediately given 5000 units of antitoxin subcutaneously and on the following day (4 days after injection) the reaction area measured 0.6 cm., while 48 hours after the administration of the antitoxin there was no sign of the reaction present. The decrease in size and the rapid disappearance of the reaction were unquestionably due to the antitoxin that was given.

The other was the case of a girl who was intensely jaundiced. On her arm the entire area of reaction was the size of a 25-cent piece, in the center of which were numerous capillary hemorrhages causing an area about the size of a dime to be bright red in color, surrounded by a brownish-yellow zone. Marked pigmentation followed. The hemorrhages resulted from the irritant action of the toxin on a jaundiced skin. The pigment was derived, in part at least, from the blood.

A 2nd outbreak of diphtheria occurred at the hospital during the month of June, 1916, and it was traced to a nurse, who had showed a positive Schick reaction in December. She passed through a typical attack of diphtheria and gave positive cultures for diphtheria bacilli. Eleven other nurses who had reacted negatively to the test also gave positive cultures for diphtheria bacilli and were quarantined, but none of these developed clinical diphtheria and none received any antitoxin.

#### CONCLUSIONS

1. In practically all cases the pigment is darker in color than the skin.
2. The reaction is equally as clearcut in negroes as it is in whites.
3. This study of 210 cases indicates that adult negroes possess about the same degree of immunity to diphtheria as do white adults.
4. Lichenification occurs in all positive cases regardless of the color of the skin and promises to be of value in differentiating positive from negative reactions in those rare cases where an increase of pigmentation is impossible.

## EFFECT OF TETHELIN ON EXPERIMENTAL TUBERCULOSIS \*

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The treatment of tuberculosis may be considered from 2 important standpoints. The one, a direct therapy, is concerned mainly in influencing the tubercle bacillus directly, that is, by killing it — a tuberculocide — or by inhibiting its growth, thus considering the influence of the host as merely passive. The other, an indirect therapy, is concerned mainly in influencing the host, which effect may be observed in the tubercle, and by this means having an indirect influence on the tubercle bacillus invading the host. Of course an ideal therapeutic agent would be one which would have both a favorable direct and a favorable indirect action at the same time. It is conceivable that a therapeutic agent may have a favorable direct action and still its unfavorable indirect action may remove it from practical consideration or vice versa. Up to the present time the scientific world has not accepted, on account of lack of conclusive evidence, a single therapeutic agent of the direct type as being of any practical value. On the other hand any number of indirect therapeutic agents are accepted as matter of fact even though the evidence of their value is based mainly on empiric clinical observations. Of these may be mentioned such agents as foods (fats, that is, butter, cod liver oil, etc.), sunlight, fresh air, absolute rest, intestinal antiseptics, etc. On account of the success thus far obtained with the indirect therapeutic procedures it is naturally to be expected that this field will be far more productive than the direct, and in the zealous search for the latter the former should not be left out of consideration by the investigator searching for therapeutic agents. It was with a view to finding an additional indirect therapeutic agent which might be of practical value that the following investigations were carried out.

Within the past year Robertson<sup>1</sup> has presented the scientific world a new substance, which he terms 'tethelin' (from *τεθελώς*, growing), precipitated from the alcoholic extract of dried anterior lobes of ox

\* Received for publication April 12, 1917.

<sup>1</sup>Jour. Biol. Chem., 24, 1916, p. 392. Ibid., p. 409.

pituitaries by the addition of  $1\frac{1}{2}$  volumes of dry ether. This precipitate from the constancy of its nitrogen and phosphorus content appears to be a chemical unit, is markedly hygroscopic, soluble in water (to the extent of about 5%), ethyl alcohol, ethyl ether, chloroform, and carbon tetrachlorid, but is insoluble in a mixture of 1 part by volume absolute alcohol and  $1\frac{1}{2}$  parts of dry ether. The average yield is about 10 mg. tethelin from each anterior lobe of ox pituitary. Although containing a iminazolyl group similar to the active substance of the posterior lobe of the pituitary body, it does not possess the characteristic physiologic activity of these substances, relatively large doses administered intravenously to rabbits producing only a slight, transient fall in blood pressure and no diuresis. The effect of tethelin on the growth of white mice resembles in every particular the effects of the administration of the whole anterior lobe with an initial (preadolescent) retardation and final (postadolescent) acceleration. Tethelin-fed mice are more compact in form and build than normal animals of the same age and their coats retain the glossy, silky appearance of the coats of young animals.

Robertson and Burnett<sup>2</sup> found that the hypodermic administration of tethelin increases markedly the rate of growth of the primary, and the tendency to form metastasis in rats inoculated with carcinoma while other alcohol-soluble extractive of the anterior lobe of the pituitary body, with the exception of the lecithin fraction, exerted no appreciable influence. Lecithin fractions from eggs had no such effect.

The following observations by Robertson,<sup>3</sup> however, seemed to indicate especially that tethelin would be productive of results as an indirect therapeutic agent in tuberculosis. He found that mice suffering from inanition and given 10 mg. of tethelin hypodermically regained weight following admission of food much more rapidly than controls not receiving tethelin, and that tissue repair, as evidenced by the healing of granulating wounds in mice, was accelerated by tethelin. If, for instance, tethelin could hasten the healing of tubercles in stimulating scar tissue formation or granulating bleeding denuded areas produced by the tuberculosis or even aid in stimulating weight increase in certain cases of tuberculous inanition its use would certainly be a valuable adjunct to the procedures at present available in treating tuberculosis.

<sup>2</sup> Jour. Exper. Med., 23, 1916, p. 631.

<sup>3</sup> Jour. Am. Med. Assn., 1916, 66, p. 1009.

To determine the value of an indirect therapeutic agent, in which class tethelin must be considered, practically 2 routes of investigation are open: to note the effect of treatment in practical doses on the rate of development of tuberculosis in the experimental animal, and to determine the effect of treatment on the local tubercle which is the true index of indirect therapeutic action.

EFFECT OF TETHELIN ON THE RATE OF DEVELOPMENT  
OF TUBERCULOSIS

In this series are included 8 male guinea-pigs of approximately the same weight. All were infected by the subcutaneous injection in the left lower quadrant of the abdomen, of 0.05 mg. of an emulsion of a virulent tubercle bacillus, No. 1305, the culture on glycerin agar being about 2 months old. Four of these pigs were used as controls not receiving tethelin and 4 of them received tethelin in 25 mg. doses in 0.5 c.c. sterile salt solution on alternate days for 18 days beginning on the day of infection. At the end of  $2\frac{1}{2}$  months those not having died before this time were killed and examined for the extent of involvement with tuberculosis. The guinea-pigs were weighed at frequent intervals during the course of the experiment, but no significant difference between the controls and test pigs was obtained.

My thanks are due Prof. T. Brailsford Robertson for supplying the tethelin used in these experiments and for his cooperation.

CONTROL ANIMAL 1.—Examination after the animal was killed,  $2\frac{1}{2}$  months after infection, revealed a small nodule at the site of injection; the local glands were slightly enlarged and caseous, the retroperitoneal glands were enlarged and hard, the spleen was about 5 times normal size and full of small necrotic areas, the liver revealed nothing macroscopically, the peribronchial and tracheal glands were markedly enlarged and hard and the lungs contained a number of small tubercles. Tuberculosis +++.\*

CONTROL ANIMAL 2.—Examination after the animal was killed,  $2\frac{1}{2}$  months after infection, revealed nothing at the site of injection, but small caseous local glands and large hard retroperitoneal glands; the spleen was 10 times normal size and full of large necrotic areas; the liver contained a few necrotic areas; the tracheal and peribronchial glands were much enlarged and hard; and the lungs contained a few isolated tubercles. Tuberculosis +++.

CONTROL ANIMAL 3.—Examination after the animal was killed,  $2\frac{1}{2}$  months after infection, revealed on section a local discharging ulcer at the site of injection, large caseous local glands, and small hard retroperitoneal glands; the spleen was 5 times normal size and contained a few large solitary necrotic areas; the liver appeared normal; the tracheal and peribronchial glands were large and hard; and the lungs contained a few isolated tubercles. Tuberculosis ++.

\* This marking is given to designate the impression of the amount of tuberculosis noted.



CONTROL ANIMAL 4.—Examination following the death of the animal, 2 months after infection, revealed a small nodule at the site of injection, fairly large caseous local glands, and large hard retroperitoneal glands; the spleen was about 10-15 times normal size and was full of miliary tubercles; the liver contained a few necrotic areas; the peritoneal cavity contained a bloody fluid; the tracheal and peribronchial glands were large and hard; and the lungs contained a few isolated tubercles. Tuberculosis + + +.

TREATED ANIMAL 1.—Examination after the animal was killed, 2½ months after infection and beginning of treatment with tethelin, revealed a small nodule at the site of injection, fairly large caseous local glands, fair-sized hard retroperitoneal glands; the spleen was about 10 times normal size and full of small necrotic areas; the liver contained a few necrotic areas; the tracheal and peribronchial glands were markedly enlarged and hard; and the lungs contained a few isolated tubercles. Tuberculosis + + +.

TREATED ANIMAL 2.—Examination after the animal was killed, 2½ months after initiation of the experiment, revealed a small local ulcer at the site of injection; the local glands were large and caseous; the retroperitoneal glands were not very large but hard; the spleen was about 5 times normal size and contained a few large necrotic areas; the liver appeared normal; the tracheal and peribronchial glands were markedly enlarged and hard; and the lungs contained a few isolated tubercles. Tuberculosis + +.

TREATED ANIMAL 3.—Examination after the animal was killed, 2½ months after initiation of the experiment, revealed a small local nodule, large caseous local glands, and large hard retroperitoneal glands; the spleen was full of small necrotic areas and about 10 times normal size; the liver contained a few necrotic areas; the tracheal and peribronchial glands were large and hard; and the lungs revealed nothing macroscopically. Tuberculosis + + +.

TREATED ANIMAL 4.—Examination after the animal was killed, 2½ months after infection and beginning treatment with tethelin, revealed a small local nodule and large caseous local glands; the retroperitoneal glands were of fair size and hard; the spleen was enlarged about 10 times normal size and contained numerous necrotic areas; the liver was practically normal; the tracheal and peribronchial glands were large and hard; and the lungs contained a few isolated tubercles. Tuberculosis + + +.

#### EFFECT OF TETHELIN ON THE DURATION OF LIFE

In this series are included 8 male guinea-pigs of approximately the same weight; all were infected by the subcutaneous injection in the lower left quadrant of the abdomen of 0.5 mg. of an emulsion of virulent human tubercle bacilli No. 7, the culture on Petroff medium<sup>4</sup> being about 11½ months old, freshly isolated from tuberculous sputum.

Four of these pigs were used as controls not receiving tethelin and 4 of them received 0.5 c.c. salt solution containing 25 mg. tethelin on alternate days for 16 days, beginning on the day of infection. The pigs were allowed to die a natural death, the duration of life and the extent of the tuberculosis at the time of death were noted.

<sup>4</sup> Jour. Exper. Med., 1915, 21, p. 38.



CONTROL ANIMAL 1.—Examination following death of animal, 62 days after infection, revealed a local ulcer; the inguinal glands were enlarged and caseous; the spleen was 5 times normal size and full of necrotic areas; the retroperitoneal glands were large and hard; the liver was large and necrotic; the tracheal and peribronchial glands were large and hard; and the lungs contained a few isolated tubercles. Tuberculosis + + + +.

CONTROL ANIMAL 2.—Examination following the death of the animal, the 136th day after infection, revealed a healed local site, slightly enlarged local glands, no enlarged retroperitoneal glands; the spleen was about 10 times normal size and full of miliary necrotic areas; the liver was full of massive necrotic areas; the tracheal and peribronchial glands were large and hard; and the lungs contained numerous fibrocaseous tubercles. Tuberculosis + + +.

CONTROL ANIMAL 3.—Examination following the death of the animal, the 105th day after infection revealed no local site but large caseous local glands; the retroperitoneal glands were large and hard; the spleen was about 5 times normal size and was full of necrotic areas; the liver was full of massive necrotic areas; the tracheal and bronchial glands were large and hard; and the lungs contained numerous fibrocaseous tubercles. Tuberculosis + + +.

CONTROL ANIMAL 4.—Examination following the death of the animal on the 82nd day after infection revealed a healed local site, large caseous local glands, and large retroperitoneal glands; the spleen was about 5 times normal size and full of necrotic areas; the liver was full of massive necrosis; the tracheal and bronchial glands were large and hard; and the lungs contained a few tubercles. Tuberculosis + + +.

TREATED ANIMAL 1.—Examination following the death of the animal, 57 days after infection, revealed a local discharging ulcer; the local glands were large and caseous; the retroperitoneal glands were slightly enlarged and hard; the spleen was 10 times normal size and full of diffuse necrotic masses; the liver was full of necrotic areas; the tracheal and peribronchial glands were slightly enlarged and hard; and the lungs contained numerous small tubercles. Tuberculosis + + +.

TREATED ANIMAL 2.—Examination following the death of the animal, 110 days after infection, revealed a healed local site; the local glands were large and caseous; the retroperitoneal glands were large and hard; the spleen was 10 times normal size and full of necrotic areas; the liver was diffusely necrotic; the tracheal and peribronchial glands were large and hard; and the lungs contained many tubercles. Tuberculosis + + +.

TREATED ANIMAL 3.—Examination following the death of the animal, 80 days after infection, revealed a healed local site; the local glands were slightly enlarged and hard; the retroperitoneal glands were large and hard; the spleen was enlarged 20 times and was full of diffuse necrosis; the liver revealed no tuberculosis; the tracheal and peribronchial glands were markedly enlarged; and the lungs contained numerous tuberculous foci. Tuberculosis + + +.

TREATED ANIMAL 4.—Examination following the death of the animal, on the 78th day after infection, revealed a healed local site; the local glands were large and hard; the retroperitoneal glands were large and hard; the spleen was enlarged 5 times and was full of diffuse necrosis; the liver contained a few small necrotic areas; the tracheal and peribronchial glands were markedly enlarged and hard; the lungs contained a few tuberculous foci. Tuberculosis + +.

As a result of these experiments it seems justifiable to conclude that tethelin in the manner (subcutaneous) and doses (25 mg. on alternate days for 16-18 days) given had no appreciable effect on the progress of tuberculosis in the guinea-pigs or on the duration of life of the animals.

#### EFFECT OF TETHELIN ON THE LOCAL TUBERCLE

Since the tubercle is the expression of the body's fight against tuberculosis it would seem that an indirect therapeutic agent if it is to be considered of any value in tuberculosis must express itself in some action on the tubercle. In order to study this action experimentally, it is necessary that a standard tubercle be produced which is easily observed at frequent intervals and possible of duplication under the same conditions of experiment. In experiments to be published later,

TABLE 1  
DEVELOPMENT AND RECESSION OF NORMAL TUBERCLE IN GUINEA-PIGS \*

Days	1A	1B	2A	2B	3A	3B	4A
2	0.17	0.37	0.16	0.28	0.18	0.31	0.23
4	0.30	0.42	0.23	0.40	0.26	0.36	0.35
6	0.34	0.43	0.31	0.40	0.33	0.37	0.39
8	0.34	(0.38)	(0.27)*	(0.38)	(0.30)*	(0.36)*	(0.35)
10	(0.26)	(0.28)	(0.23)	(0.20)	(0.25)	(0.25)	(0.27)
12	(0.22)	(0.28)	(0.13)	(0.20)	(0.22)	(0.22)	(0.23)
14	(0.21)	(0.26)	(0.12)	(0.16)	(0.16)	(0.19)	(0.20)
16	(0.20)	(0.18)	(0.12)	0.10	(0.15)	0.14	0.20
18	(0.15)	(0.16)	(0.11)	0.09	0.15	0.10	0.14
20	(0.14)	0.14	0.10	0.09	0.12	0.08	0.12
22	(0.13)	0.13	0.09	0.08	0.10	0.08	0.10
28	0.08	0.09	0.06	0.06	0.07	0.06	0.08
Weight at beginning of exper.	905	850	850	795	990	990	795

\* The (b) tubercles were initiated 23 days after injecting the bacilli intracutaneously for the (a) tubercles.

Guinea-pigs 1, 2, 3, and 4 were given the previous subcutaneous injection of living avirulent bacilli, while 5, 6, 7, and 8 were given the dead bacilli.

The diameter of the tubercles though taken are of no especial significance and are therefore omitted.

The numeral in parentheses signifies that the tubercle has ruptured and is discharging. The asterisk after such numeral indicates that only 1 tubercle has ruptured at the time recorded.

it has been found that such tubercles may be produced by the intracutaneous injection of standard amounts of dead tubercle bacilli into the abdomen of guinea-pigs, which had previously received a primary injection of either dead or living tubercle bacilli. There was a slight difference in the tubercles thus produced in different guinea-pigs, but in the same guinea-pig it was possible to duplicate, with regard to development and rupture, the tubercle at least 3 times. Thus if the 1st intracutaneous tubercle was used as the control, the effect of the

therapeutic agent to be tested could be gauged on the 2nd or 3rd intracutaneous tubercle produced.

In this series of experiments are included 8 male guinea-pigs, 4 of which (5, 6, 7, and 8) had been given an initial injection of dead human tubercle bacilli equivalent to 0.2 mg. of water free (dried at 100 C. for analysis) residue 52 days previous to initiation of the test, and 4 of which (1, 2, 3, and 4) had been given an initial injection of 5 mg. of living avirulent human tubercle bacilli 221 days before the initiation of this test. Two of the former set and 2 of the latter set of pigs were used as controls (that is, carried through entirely without treatment) while the remaining 2 of each set were used to gauge the effect of the tethelin. In all of them, however, the 1st intracutaneous tubercles, of which there were 2 produced in each animal (the mean of these being given in the tables), were used as the controls without treatment with tethelin. The intracutaneous tubercles were produced by the injection of 0.1 c.c. of an emulsion of dead human tubercle bacilli containing an equivalent of 0.11 mg. of bacillary bodies dried at 100 C. (The dry weight was used merely as the gauge of amounts.)

TABLE 1—*Continued*  
DEVELOPMENT AND RECESSION OF NORMAL TUBERCLE IN GUINEA-PIGS \*

4B	5A	5B	6A	6B	7A	7B	8A	8B
0.28	0.17	0.30	0.19	0.28	0.29	0.34	0.20	0.32
0.39	0.29	0.42	0.29	0.36	0.42	0.42	0.27	0.37
0.42	0.29	(0.40)*	0.33	0.42	0.42	0.42	0.29	0.38
(0.38)	(0.25)*	(0.32)	(0.26)	(0.32)	(0.42)*	(0.44)	(0.25)*	(0.31)
(0.21)	(0.25)	(0.21)	(0.19)	(0.24)	(0.28)	(0.36)	(0.19)	(0.24)
(0.17)	(0.23)	(0.17)	(0.15)	(0.18)	(0.24)	(0.30)	(0.15)	(0.17)
0.12	(0.22)	(0.16)	(0.13)	(0.15)	(0.21)	(0.26)	(0.13)	(0.17)
0.10	(0.21)	0.11	0.13	0.10	(0.18)	(0.21)	(0.13)	(0.11)
0.08	0.20	0.09	0.11	0.08	(0.17)	(0.17)	(0.12)	0.09
0.08	0.20	0.07	0.10	0.07	0.15	0.14	0.10	0.09
0.07	0.09	0.06	0.08	0.06	0.12	0.11	0.10	0.08
0.06	0.07	0.04	0.06	0.05	0.08	0.09	0.08	0.06
825	765	740	710	740	655	655	680	655

The figures in the tables are given in centimeters of actual tubercle thickness as measured by calipers graduated in one hundredth of a centimeter, the figure being obtained by subtracting from the mean figure, obtained by measuring the tubercle plus twice the skin thickness, the thickness of the double normal skin as control in the vicinity of the tubercle, but far enough away not to be influenced by it. The 8 animals referred to by number in Table I were used for the experiments in Tables 2 and 3. In Table 2 it is noted that one half of the animals (the even numbers) were treated with tethelin while the other half (odd numbers) were not treated during observation of the tubercle. In Table 3 the treatment with tethelin was continued (with the even numbers), while the wound healing was studied in the entire series.

Table 1 shows 2 important facts: The development and recession, including healing of the tubercle, produced by the intracutaneous injection of dead human tubercle bacilli in previously sensitized guinea-pigs, occurs in a fairly definite curve form, although there are distinct variations in size of the individual tubercles thus produced; and the tubercle ruptures in these animals at a fairly definite time—in the

TABLE 2  
DEVELOPMENT AND RECESSION OF TUBERCLE UNDER TREATMENT WITH TETHELIN \*

Days	1	2	3	4	5	6	7	8
2	0.20	0.27	0.27	0.24	0.18	0.17	0.30	0.22
4	0.29	0.33	0.31	0.36	0.26	0.31	0.36	0.24
6	0.34	0.36	0.37	0.42	0.33	0.34	0.38	0.28
8	0.35	(0.26)	(0.38)	(0.35)	(0.26)	(0.24)	(0.30)	0.28
10	(0.28)	(0.16)	(0.19)	(0.18)	(0.23)	(0.18)	(0.21)	(0.20)
12	(0.15)	(0.14)	(0.16)	(0.16)	(0.21)	(0.13)	(0.17)	(0.18)
14	(0.13)	(0.12)	0.12	(0.12)	(0.20)	(0.12)	(0.16)	(0.15)
16	(0.13)	(0.10)	0.11	0.10	0.08	(0.11)	(0.14)	(0.13)
18	(0.11)	0.07	0.10	0.08	0.06	0.09	(0.13)	(0.11)
20	0.09	0.06	0.08	0.06	0.05	0.08	0.12	0.08
22	0.08	0.05	0.07	0.06	0.05	0.07	0.09	0.07
28	0.04	0.03	0.05	0.04	0.03	0.05	0.06	0.04
Weight in grams	905	825	1050	850	765	795	710	655

\* These intracutaneous tubercles were initiated 16 days after tubercle (b) of Table 1 had been initiated and the figures are the mean of readings of 2 tubercles, after deducting the double skin thickness as in Table 1. The even number animals (2, 4, 6, and 8) received 25 mg. doses of tethelin in 0.5 c.c. physiologic salt solution practically daily from 4 days previous to the initiation of the tubercle until 18 days after (a total of 0.4 gm. to each animal) when it was further continued as stated in footnote to Table 3.

The numbers of the animals correspond to those in Table 1.

TABLE 3  
HEALING OF DEEP SKIN WOUNDS UNDER TETHELIN TREATMENT \*

Days	1	2	3	4	5	6	7	8
0	0.45	0.45	0.47	0.46	0.46	0.46	0.48	0.44
1	0.40	0.34	0.43	0.44	0.42	0.48	0.47	0.43
2	0.35	0.34	0.48	0.45	0.39	0.47	0.47	0.42
4	0.34	0.33	0.47	0.44	0.40	0.42	0.41	0.40
6	0.28	0.32	0.41	0.43	0.35	0.37	0.38	0.32
8	0.25	0.27	0.35	0.34	0.20	0.15	0.21	0.18
10	—†	0.18‡	0.29	0.25‡	0.16‡	0.13	0.12	0.15‡
12	—†	—†	—†	0.10‡	—†	—†	—†	0.09‡
Weight at end of exper. in grams	825	825	1080	850	710	825	680	680

\* These animals are a continuation of the ones referred to in Tables 1 and 2, the numbers corresponding, and the administration of tethelin was continued to the even numbers in 25 mg. doses daily in 0.5 c.c. physiologic salt solution (having been given a total of 0.4 gm. in 22 days) for another 10 days, an additional total of 0.25 gm. more.

These figures are diameter of lesion in centimeters, a mean of 2 figures, that is, 2 lesions; wherever the lesion was irregular in shape the mean of the longest and shortest diameters is used as the individual reading of a lesion.

The holes were made by lifting up a double fold of skin on the abdomen of the guinea-pig after shaving and cleaning thoroughly and punching a double hole through the entire by means of a sharp paper punch making round perforations about 0.4-0.5 cm. in diameter.

† A dash indicates the lesion is entirely healed and only a contracted scar tissue remains.

‡ A double dagger after a figure indicates that only one lesion is healed.



above experiments the 8th or 10th day (as will be shown in a subsequent paper, this rupture may be influenced by at least 1 factor, the nutrition of the animal).

Table 2 shows that the administration of tethelin in 25 mg. doses daily to 650-850 gm. sensitized guinea-pigs had no influence either on the development or recession (and healing) of the tubercle or on its rupture.

Table 3 shows that tethelin in 25 mg. doses daily given to 680-850 gm. sensitized guinea-pigs had no appreciable effect on the healing of deep wounds.

#### DISCUSSION

Robertson<sup>3</sup> obtained a hastening of wound healing in mice weighing about 20-25 gm. after administration of 10 mg. of tethelin, which would correspond to a dosage of at least 200-400 mg. per 500-900 gm. guinea-pig, and to prohibitively large doses for human beings while in the above experiments reported only 25 mg. were used daily. The mice used by Robertson were also normal, and taking into consideration the difference in dosage, the fact that different animals were used, and that these animals were sensitized to tuberculosis, it is easily appreciated that while his experiments were productive of results these experiments, differing as they do, are in no way contradictory to his findings—the conditions being different. From a practical standpoint, however, it would be impossible to use in tuberculosis a dose of tethelin even approaching that used in the foregoing experiments. For this reason and unless the human being is much more susceptible in response toward tethelin it would hardly seem that tethelin in practical doses can have any effect by analogy on tuberculosis as found in the human being. The foregoing experiments also do not rule out the possibility of tethelin in very large doses having a favorable action on the tubercle, since this was not tried. It may someday be possible to produce tethelin synthetically or to further concentrate the wound-healing principle which might still make it practically applicable as an indirect therapeutic agent in tuberculosis. At present, however, its administration can hardly be justified as a therapeutic agent.

#### SUMMARY

Tethelin the active principle of the anterior lobe of the pituitary, administered subcutaneously in 25 mg. doses on alternate days for 18 days before and during early infection to tuberculous guinea-pigs,



infected with virulent human tubercle bacilli, had no appreciable effect on the progress of the tuberculosis or on the duration of life of these animals.

Tethelin administered subcutaneously in 25 mg. doses daily to guinea-pigs sensitized to tuberculosis by dead and living (avirulent) human tubercle bacilli had no appreciable effect on the development, recession, or rupture of intracutaneous tubercles produced by dead human tubercle bacilli nor on deep puncture wounds of the skin in these animals.

## PRECIPITIN-PRODUCTION IN ALLERGIC RABBITS \*

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In the course of efforts to develop in rabbits by the intraperitoneal injection of large quantities of horse blood or horse serum precipitins which would detect minute quantities of horse protein, it was observed that the introduction of horse blood or serum in rabbits treated a considerable time previously with sheep blood resulted in the production not only of precipitin for horse protein, but also for sheep protein, as well as for proteins of several other species. The wide range of the precipitating action of the serum rendered it quite unsuitable for the detection of horse protein in the presence of proteins from various other sources. Unless carefully and thoroughly studied, the use, for precipitin tests, of serum obtained under these and similar conditions might lead to highly misleading results.

These statements are based on a number of observations, some of which may be given in detail by way of illustration:

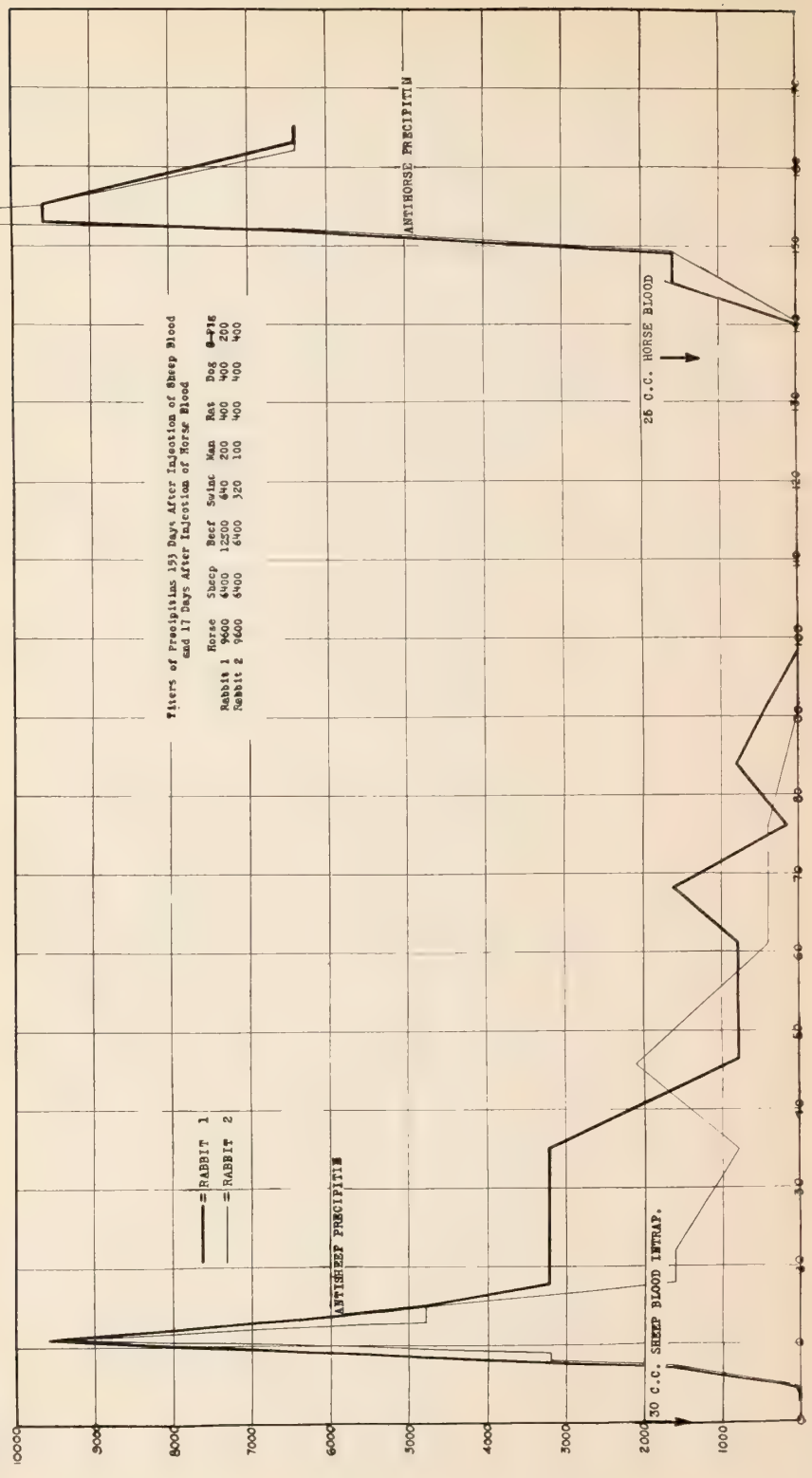
1.—Two rabbits each received 30 c.c. of sheep blood intraperitoneally on Sept. 23, 1915. Both developed a goodly quantity of precipitin for sheep blood as shown in Chart 1. Here as in the other charts the titer gives the highest dilution in salt solution of laked blood—sheep, horse, etc.—with restored salt content, in contact with which 0.1 c.c. of rabbit serum gave a definite precipitate after 1 hour at room temperature. The curves are quite typical: The highest concentration was reached about the 11th day after the injection and by about the 90th day—December 23—the serum appeared to have lost its precipitating effect. Tests with other protein solutions than sheep blood were not made during this time. On Feb. 8, 1916, 25 c.c. of horse blood were injected intraperitoneally and there developed, as shown also in Chart 1, precipitin in high concentration for horse blood, the dilutions of the horse blood and the tests being made in the same way as in the case of the sheep blood. Tests were now made in the same manner with the blood of other species and the results of 1 test are given on the chart, the figures representing, as stated, the highest dilutions of blood in which precipitates appeared. Several other tests were made with practically the same outcome.

We see that in these 2 rabbits the injection of horse blood some 5 months after the sheep blood, and several weeks after the precipitin for sheep blood had disappeared from the serum, resulted in the development of precipitins not only for horse blood, but also in about

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FIG. 2

Highest dilution  
of blood in which  
serum caused pre-  
cipitate



DAYS

Chart 1.—Precipitins in 2 rabbits injected with sheep blood, and 140 days later with horse blood.

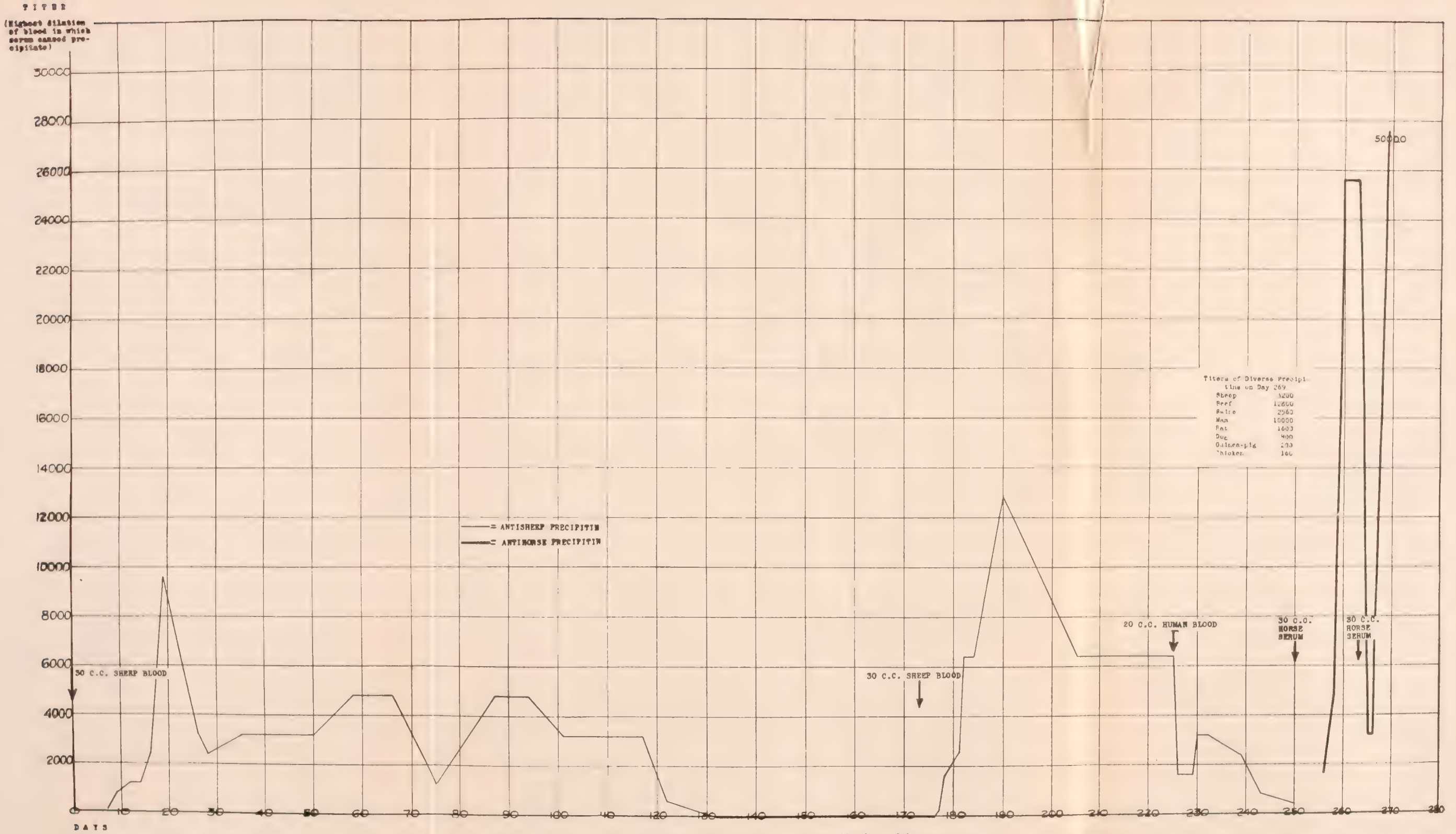


Chart. 2.. Precipitins in rabbit injected with sheep blood, human blood, and horse serum.



TITERS  
(Highest dilution  
of blood in which  
serum caused pre-  
cipitate and lysis.)

30000  
28000  
26000  
24000  
22000  
20000  
18000  
16000  
14000  
12000  
10000  
8000  
6000  
4000  
2000  
0

— ANTISHEEP PRECIPITIN  
— ANTIRHAB PRECIPITIN  
- - - ANTIRHAB PRECIPITIN  
- - - ANTIRHAB LYSIN

TITERS OF PRECIPITINS OTHER THAN THOSE CHARTED

Days After 2nd Sheep Blood Infection of	Man	Rat	Dog	Guinea Pig
54	540	200	400	40
57	1750	800	800	40
60	5120	1200	800	
74	2560	800	1600	1600
88	1280	800	100	40
94	1280	600	0	10
97	Infection of 10 c.c. of guinea-pig serum			
100	80	400	0	150
104	0	0	0	200
108	Infection of 10 c.c. of sheep serum			
107	0	100	0	200
114	0	1600	400	0
118	120	16	0	0
121	80	0	400	1600
124	2560	800	1600	400
128	2560	800	1600	400
131	2560	800	1600	400
134	2560	800	1600	800
136	Infection of 10 c.c. of sheep blood			
140	0	0	0	0
144	0	0	0	0
147	1200	1600	800	1600
151	1200	1600	800	1600
154	6400	1600	12800	800
158	2560	800	800	0
161	2560	800	400	0
179	2560	800	400	0
181	640	400	100	0

30 c.c. SHEEP BLOOD  
TOLUENE 1 c.c. PER K  
DAILY FOR 10 DAYS  
BEFORE THIS INJECTION

30 c.c. SHEEP BLOOD

BENZENE PER K  
50 c.c.

50 c.c. HORSE SERUM

10 c.c. GUINEA PIG SERUM

50 c.c. HORSE SERUM

50 c.c. SHEEP BLOOD

49000  
49000

50000

0 9 19 29 39 49 59 69 79 89 99 109 119 129 139 149 159

Chart 3. Lysin and precipitins in rabbit injected with sheep blood, horse serum, etc.



equal degree for sheep and beef blood, and, though in much less degree, for blood of the other species tested.

That the previous stimulation of precipitin-production by sheep blood is largely responsible for the development of so many precipitins after the injection of horse blood, as illustrated in the foregoing, is indicated by the rather strict specifcness of the precipitin which develops after the injection of foreign blood in previously untreated animals. Thus in the case of a series of 6 fresh rabbits injected intraperitoneally with 30 c.c. of horse serum, there developed in 4 precipitin waves, which at the height gave titers of 6400-12800 with horse blood and no precipitates at all in dilutions above 1:50 with the blood of any of the other species discussed in this article; and, as is well known, results like this are usually obtained from the injection of untreated rabbits with the serum of blood of other species. I have observed, however, that in the fresh rabbit the injection of sheep blood sometimes may result in the production of considerable precipitin for beef blood as well as for sheep blood.

2.—A rabbit, weighing 1050 gm., received 30 c.c. of sheep blood intraperitoneally on May 12, 1915. At the same time it received 1 c.c. of toluene in 1 c.c. of olive oil subcutaneously and this was repeated daily for 12 days. Precipitin for sheep blood developed, the concentration being somewhat less and the fluctuation greater than in the previous case, but the persistence was longer—until somewhere between 122 and 132 days after the injection of the sheep blood.<sup>1</sup> On Day 173 (October 20) 30 c.c. of sheep blood were injected intraperitoneally again and, after a latent period of 3 days, a new wave of precipitin-production set in, reached its high point (12800) about the 12-17th day (Chart 2) after the 2nd injection, and maintained a high level for about 30 days, when 20 c.c. of human blood were injected intraperitoneally, after which it declined rather abruptly. In the early part of January, 1916, about 70 days after the 2nd injection of sheep blood and 243 days after the 1st injection, the precipitin titer was 800 with sheep blood. Practically no precipitin for human blood developed at this time. On Day 250 30 c.c. of horse serum were injected intraperitoneally and abundant precipitin for horse blood developed, the titers on the 10th, 11th, and 12th days being 25-50000+. Thirteen days later the injection of horse serum was repeated and there resulted a marked drop in the titer, but in a few days however, the titer was again 50000+ for horse blood, and tests with blood of other species now gave the following results: sheep, 3200; beef, 12800; swine, 2560; man, 10000; rat, 1600; dog, 400; guinea-pig, 200; chicken, 160.

In this case an especially noteworthy feature appears to me to be the stimulation by the injection of horse serum of the elaboration of precipitin for human blood, the injection of 20 c.c. of which some

<sup>1</sup> This rabbit is the same as Rabbit 5, Table 2, in the article, "The Effect of Toluene on the Production of Antibodies," Jour. Infect. Dis., 1916, 19, p. 737.

weeks previously had been without hardly any recognizable precipitinogenic effect. In this animal as well as in others the titer of the anti-beef precipitin runs especially high.

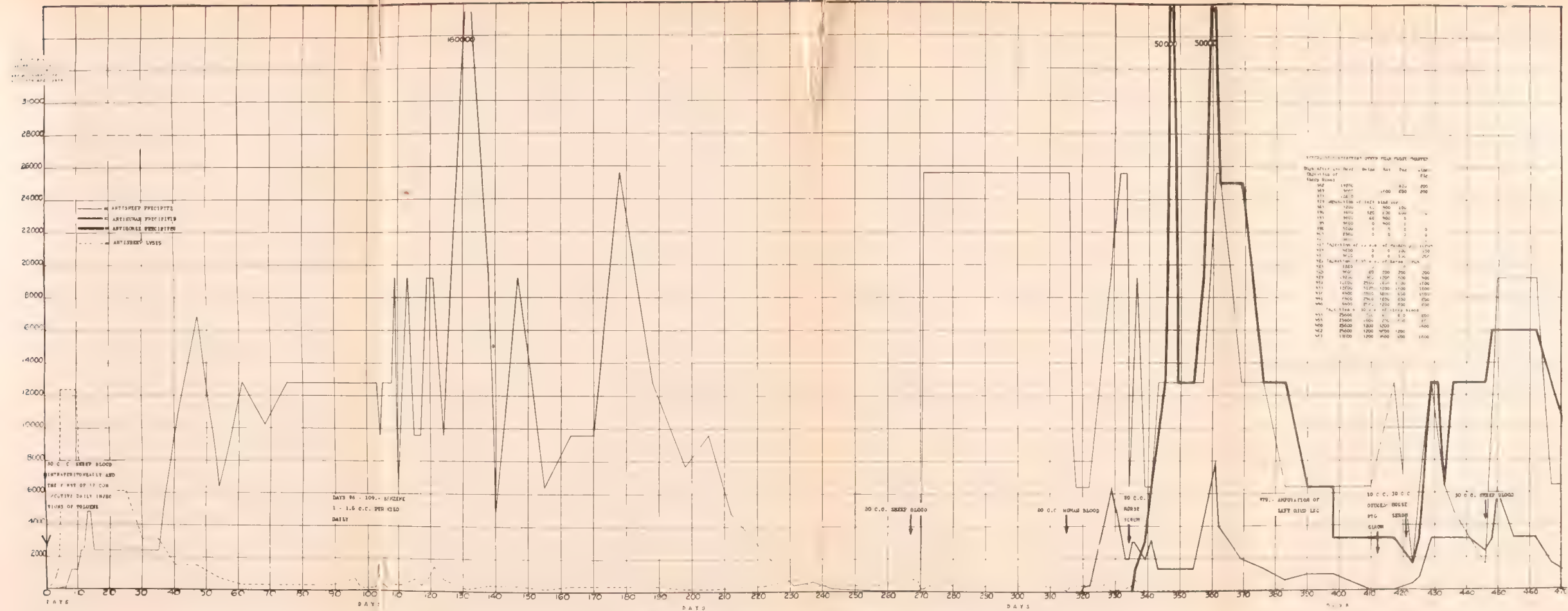
3.—The rabbit now considered was 1 of a group which received 10 or more daily injections of toluene 1 c.c. per kilogram of weight before the injection of 30 c.c. of sheep blood intraperitoneally.<sup>2</sup> It developed not even a trace of precipitin, and 171 days after this injection it was given the same amount of sheep blood whereon much precipitin accumulated in the blood in spite of the fact that on the 7th, 8th, 9th, and 10th days after the 2nd injection it was given 1.5 c.c. per kilogram of benzene subcutaneously, and on the 11th and 12th days, 2 c.c. (Chart 3). On the 47th day after the 2nd injection of sheep blood it was given 30 c.c. of horse serum intraperitoneally; on the 96th day, 10 c.c. of guinea-pig blood; on the 105th day, 30 c.c. of horse serum again; and finally on the 136th day, 30 c.c. of sheep blood. Chart 3 gives the results of the observations on precipitins and antisheep lysin.

We note that the beef precipitin, which with the precipitins that are not charted was not estimated until 40 odd days after the second injection of sheep blood, follows quite closely the general course of the sheep and horse precipitins as influenced by injections of horse serum and sheep blood. As set forth by the figures on Chart 3, the injection of guinea-pig serum was succeeded by a wave of specific precipitin which subsided on the injection of sheep blood only to reappear in about 10 days; the other precipitins followed a like course, but the antisheep lysin runs quite independently and seems not to have been influenced by any other antigen than sheep blood.

4.—A young rabbit, weighing about 1000 gm., was injected with 30 c.c. of sheep blood intraperitoneally and 1 c.c. toluene in olive oil subcutaneously. The toluene injection was repeated daily for 12 days in order to study its effect on antibody-production. The subsequent injections and the results of the observations on the antibody-content of the serum are shown in Chart 4. The rabbit increased in weight until the 243rd day when it weighed 2025 gm.; thereafter there was more or less fluctuation and decline. The injections of benzene on Days 96-109 had no effect on the leukocytes or, as far as apparent, on the lysin and precipitin, the remarkably prolonged persistence of which with great fluctuation as to concentration in the serum may be ascribed at least in part to the effects of the injections of toluene.<sup>3</sup> On the 267th day 30 c.c. sheep blood were injected again and there resulted a prompt and marked increase in antisheep precipitin but not in lysin, which reached normal level while the precipitin was still present in large quantities, its content in the serum being influenced greatly by the subsequent injections of human blood, guinea-pig serum, horse serum, and sheep blood. As a rule there was a fall after each injection succeeded by a rise in all the precipitins considered. No explanation can be advanced for the fall about the 350th day. On the 379th day the left hind leg was removed in order to study the marrow; at this time the

<sup>2</sup> Rabbit 1, Table 3, in the article on toluene (<sup>1</sup>).

<sup>3</sup> This animal is Rabbit 2, Table 2, in the article on toluene (<sup>1</sup>).





blood gave 14,600 leukocytes, 47.5% being granular and 52.5% nongranular. The marrow of the amputated bones seemed about normal except for a rather large number of erythroblasts; there was no increase in eosinophils, giant cells, or kinetic figures.

The extreme divergence in the course of the antisheep lysin and antisheep precipitin in this case points to their being independent substances produced by distinct mechanisms. Johnson<sup>4</sup> records an observation of perhaps similar import in the case of rabbits injected with beef serum, and Gay<sup>5</sup> finds that the lysin in a serum containing precipitin is not brought down on the formation of the specific precipitate, thus showing quite conclusively that the substances in question are distinct.

It is not necessary to try to analyze more minutely than already done the fluctuations in the precipitin and lysin content of the serum of the rabbits under study. The particular point on which special stress is placed now is the capacity of the rabbit under suitable conditions, as shown plainly enough by the charts, to elaborate different precipitins at the same time. It appears that the precipitin-production induced in the usual way leaves behind it an increased power of further production so that large amounts of major as well as group and minor precipitins are elaborated on the injection of a new serum or blood. Manifestly the phenomenon is an expression of an increased reactivity and may be classed with other manifestations of allergy.

Whether the treatment of some of the rabbits with toluene was of much significance in respect to the degree of increased reactivity probably cannot be determined, although such may possibly have been the case; the first observation, however, shows that rabbits not treated with toluene also may develop much increase in the power to elaborate precipitins as the consequence of having passed through a previous course of precipitin-production. I find that in dogs not subjected to the action of toluene, but previously injected intravenously with a single dose of goat blood, the injection of rat blood after the new goat lysin has disappeared may cause a second wave of goat lysin at the same time as the formation of agglutinin for rat corpuscles runs its typical course. Furthermore, in dogs treated with rat blood, injection of goat blood may reawaken the production of rat agglutinin. These results are not obtainable regularly; in an experiment involving 8 dogs, 2 gave the results indicated in Table 1. It should be noted that in this case an

<sup>4</sup> Jour. Immunol., 1916, 1, p. 397.

<sup>5</sup> Ibid., p. 83.



altered reactivity is manifested by a resumption of the output of lysin and agglutinin, hence the change is not limited to the renewed production of any particular antibody. This is shown further by the experiments of Conradi and Bieling<sup>6</sup> on rabbits injected with typhoid bacilli; here subsequent injections with colon, dysentery, or diphtheria bacilli resulted in an increase in typhoid agglutinins, and in rabbits injected with paratyphoid bacilli, injection of typhoid bacilli some months later caused rapid increase in paratyphoid agglutinin. We may conclude then that the kind of increased reactivity here discussed is not limited to any particular species or antibody, and that toluene is not essential for its establishment.

TABLE 1

REACTIVATION OF LYSIN- AND AGGLUTININ-PRODUCTION IN THE DOG BY INJECTION OF BLOOD OTHER THAN THAT FIRST INJECTED \*

Days After Second Injection	First Injection, 1 C.c. 10% Suspension of Goat Blood per Kilogram; Second Injection, 1 C.c. 10% Suspension of Rat Blood per Kilogram	First Injection, 1 C.c. 10% Suspension of Rat Blood per Kilogram; Second Injection, 1 C.c. 10% Suspension of Goat Blood per Kilogram		
	Titer of Lysin for Goat Corpuscles	Titer of Agglutinin for Rat Corpuscles	Titer of Agglutinin for Rat Corpuscles	Titer of Lysin for Goat Corpuscles
2	96	56	96	96
4	384	384	1536	384
6	1536	1536	6144	384
8	1536	1536	6144	384
10	1536	1536	6144	384
13	1536	384	3072	384
17	768	192	1536	192
21	192	192	1536	192
31	48	48	768	96
41	48	48	768	96

\* The titer gives the highest dilution in which the serum produced lysis and agglutination. Concerning details of tests see Hektoen and Carlson, Jour. Infect. Dis., 1910, 7, p. 319.

These observations are, of course, not without clinical and practical interest. Most recently Conradi and Bieling,<sup>6</sup> in a study of the diagnostic value of the agglutinin test in patients inoculated against typhoid fever, cite numerous examples of marked increase in typhoid agglutinins in patients so inoculated at some previous time, but suffering from nontyphoid infections, for example, miliary tuberculosis, malaria, pneumonia, and erysipelas. It appears as if almost any infection may cause increase in typhoid agglutinin in the inoculated (and in those who have had typhoid fever)—the new infection calls forth agglutinins like those of the past, and under these circumstances a positive agglutination test may not signify typhoid fever at all. Undoubtedly, analogous conditions may arise in human and animal disease with

<sup>6</sup> Deutsch. med. Wchnschr., 1916, 42, p. 1280.

respect to other bacteria than the typhoid bacillus<sup>7</sup> and to proteins other than those of bacterial origin, but at this time it was not the intention to carry the discussion further than to make clear the general bearing of the results from the rabbit experiments.

As stated in the beginning, the serum of rabbits subject to injection of more than 1 kind of serum or blood may come to contain many precipitins in a concentration that far exceeds the limits of quantitative specificity necessary for practical tests as usually conducted. It would seem that as the blood and serum used as antigens in the rabbit experiments contain many different antigenic substances, some of which perhaps are present though in small quantities in all the species concerned, the biologic specificity of the precipitin reaction may become less pronounced, less sharply limited quantitatively, the higher the increased reactivity of the rabbit yielding the precipitin-serum. On this account the serum for practical precipitin tests should be obtained by specific immunization of suitable previously unused rabbits. On the other hand, under certain circumstances it may be found desirable in order to produce antibodies that are not easily put forth, to use allergic animals.

While the injection of foreign blood or serum into a rabbit previously injected with some other blood or serum, may call forth various precipitins in large amounts because of the many antigens in the materials which are injected into an allergic organism, it is also possible that in the allergic animal the course of immunization may have left the mechanisms of antibody-production in such state that they can be aroused again into the same activities as before by new but totally different antigenic stimuli, and possibly even by stimuli of nonantigenic nature. The experiments so far, however, in which efforts have been made to reawaken precipitin-production by means of antigens, regarded as different from that already injected, and by means of metallic colloidal suspensions have given negative results.

#### SUMMARY

In rabbits previously injected with foreign blood or serum the subsequent injection of a different blood or serum may reawaken the production of precipitins for the antigens first injected, and the serum of

<sup>7</sup> In connection with this question see Dreyer and Inman: The Agglutination Curve and Its Importance in the Diagnosis of Typhoid and Paratyphoid Fevers in Inoculated Persons, *Lancet*, 1917, 192, p. 365. Baehr: Agglutination in Typhus Fever, *Jour. Infect. Dis.*, 1917, 21, p. 21.

such rabbits may be unsuitable for practical precipitin tests because of the wide range of its action.

Similar manifestations of an increased reactivity may be obtained in dogs as regards lysin and agglutinin for goat and rat corpuscles, respectively, as well as in rabbits and human beings with respect to typhoid agglutinins, and the increase of typhoid agglutinins in infectious conditions in persons previously inoculated or infected with typhoid bacilli may not signify typhoid fever.

# A MODIFICATION OF THE McCRADY METHOD OF THE NUMERICAL INTERPRETATION OF FERMENTATION-TUBE RESULTS\*

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In the recent report of the American Public Health Association, issued in 1917, on the standard methods of bacteriologic water analysis, it is recommended that the numerical interpretation of fermentation-tube results in the determination of *B. coli* and allied bacteria should be based on the method originally outlined by Phelps.<sup>1</sup> More exact procedures of arriving at the most probable number of bacteria from qualitative results only were not favorably reviewed in the above report, since their application "to a correct mathematical solution of any considerable series of results involves mathematical calculations so complex as to be impracticable of application in general practice."

Among the methods proposed for such numerical interpretations is the one described and completely analyzed by McCrady.<sup>2</sup> McCrady bases his consideration of the problem on the fact that "the frequency of the appearance of particular fermenting organisms is an exponential function of the number of such organisms in the sample tested and that every fermentation-tube result, whether simple or compound, corresponds to one most probable number of organisms." In his discussion, he makes use of a general probability formula, by means of which the current fermentation-tube results may be converted into numerical values. The justification of McCrady's method has been amply proved, but, heretofore, their widespread use has been restricted on account of the cumbersome calculations involved when any but recurring series of tube dilutions are employed and also by the inherent dislike of most bacteriologists for apparently involved mathematical deductions.

Inasmuch as we were seeking an interpretation of qualitative results which would give numbers of bacteria which might approach a greater degree of precision than the usual method of "averages" now commonly employed, some study was made of the methods proposed by

\* Received for publication April 30, 1917.

<sup>1</sup> Am. Pub. Health Assoc., Report 33, p. 9.

<sup>2</sup> Jour. Infect. Dis., 1915, 17, p. 183.

McCrady in the hope of so modifying them as to make their application simple and consequently of greater frequency.

The formula proposed by McGrady for the calculation of the most probable number of *B. coli* is as follows:

$$(p + q) (\log .9) + (r + s) (\log .99) + (t + u) (\log .999) = \\ \frac{p (\log .9)}{1 - .9^x} + \frac{r (\log .99)}{1 - .99^x} + \frac{t (\log .999)}{1 - .999^x}$$

in which  $(p + q)$ ,  $(r + s)$ , and  $(t + u)$  are the total number of tubes inoculated in 10, 1, and 0.1 c.c. dilutions, while  $p$ ,  $r$ , and  $t$  are the number of tubes found to be positive in each of the above series.

The solution of the above equation involves the trial substitution of possible values of "x" (the most probable number of bacteria per 100 c.c.) until the equation is found to balance. The corresponding value of "x" for this condition is the value we are seeking for any possible combination of fermentation-tube results. In examining the equation, we find that:

$$\begin{aligned} \log .9 &= .04576 \\ \log .99 &= .00436 \\ \log .999 &= .000435 \\ \log .9999 &= .0000434 \end{aligned}$$

For practical purposes, it may be stated, therefore, that approximately

$$\log .9 = 10 \log .99 = 100 \log .999 = 1000 \log .9999$$

and the formula above reproduced becomes, with this substitution:

$$100 (p + q) + 10 (r + s) + 1 (t + u) = \frac{100 p}{1 - .9^x} + \frac{10 r}{1 - .99^x} + \frac{t}{1 - .999^x}$$

knowing the possible values of  $(p + q)$ ,  $(r + s)$ ,  $(t + u)$ ,  $p$ ,  $r$ , and  $t$ , it becomes a matter of little mathematical calculation to establish the value of "x" by trial solutions.

In order to make the solution of this modified formula still more practical and more facile, the values of  $1 - .9^x$ ,  $1 - .99^x$ , and  $1 - .999^x$  were graphically plotted on the attached chart. The curves, correlating values of "x" with the values of  $1 - .9^x$  etc., are exponential curves having exactly the same form and value as those given by McGrady, showing the most probable number of *B. Coli* per 100 c.c. with varying percentages of samples showing positive tests in 10, 1, 0.1 c.c. samples, respectively. By using the two scales indicated in our charts, it is made to serve two purposes; firstly, the interpretation of single dilution results, and, secondly, readings for actual calculations of values of  $1 - .9^x$ , etc., for possible trial values of "x." The accuracy of these



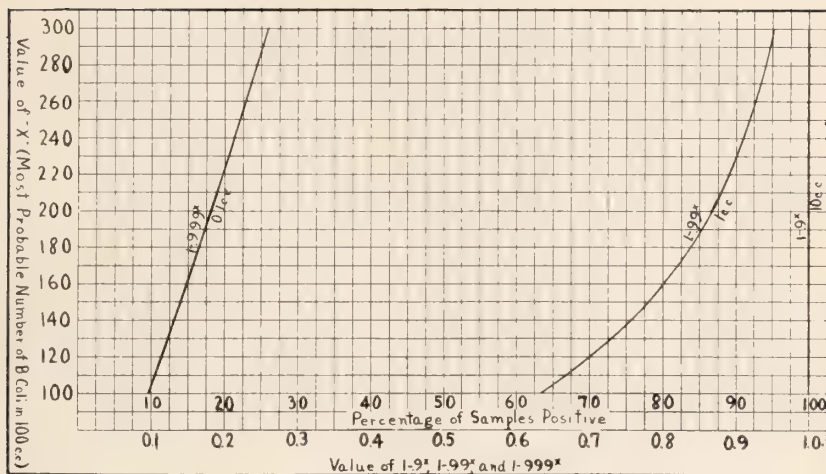


Chart 1.—Chart showing values of "X" (the most probable number of B. coli per 100 c.c.), and 1—.9%, 1—.99% and 1—.999% for varying conditions.

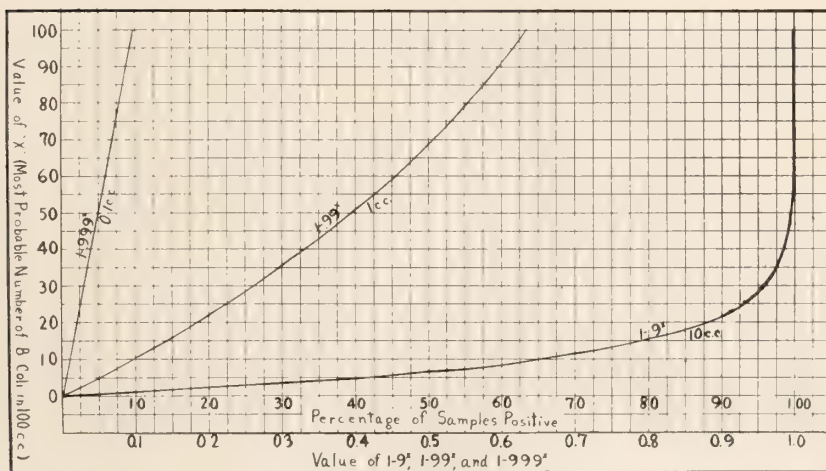


Chart 2.—Chart showing values of "X" (the most probable number of B. coli per 100 c.c.), and 1—.9%, 1—.99% and 1—.999% for varying conditions.

charts can, of course, be made as high as desired, and it is McCrady's opinion (by letter) that the error involved in the use of the curves is slight and that the method is certainly sufficient for practical use, especially for the ordinary ranges of the value of "x."

For purposes of clarification of the above method, it is well to solve several typical examples, as follows:

*Single Dilution:* Given the result 4 out of 5 positive in 10 c.c., to find the most probable number of *B. coli* in 100 c.c.

4/5 in 10 c.c. is equivalent to 80% positive.

Using this value of 80 as an ordinate, with the 10 c.c. curve on Chart 1, we find the corresponding value of the abscissa to be 15. The most probable number of *B. coli* per 100 c.c. is, therefore, 15, for the condition 4/5 in 10 c.c.

*Several Dilutions:* Suppose the result of the fermentation tubes gives positive values of  $\frac{1}{2}$  in 10 c.c.,  $\frac{3}{10}$  in 1 c.c., and  $\frac{1}{10}$  in 0.1 c.c., the above equation becomes:

$$100 (2) + 10 (10) + 1 (10) = 310 = \frac{1 (100)}{1 - .9^x} + \frac{10 (3)}{1 - .99^x} + \frac{1}{1 - .999^x}$$

Using trial value of  $x = 22$ , by referring to the chart we have:

$$1 - .9^x = .90 \quad 1 - .99^x = .195 \quad \text{and} \quad 1 - .999^x = .02$$

$$\text{Therefore: } 310 = \frac{100}{.90} + \frac{30}{.195} + \frac{1}{.02} = 315$$

Using trial value of  $x = 23$ :

$$1 - .9^x = .91 \quad 1 - .99^x = .205 \quad 1 - .999^x = .02$$

$$\text{Therefore: } 310 = \frac{100}{.91} + \frac{30}{.205} + \frac{1}{.02} = 308$$

The probable number of *B. coli* per 100 c.c. is, therefore, between 22 and 23.

It has probably already become clear to the reader that the divisions carried out above may be performed generally with sufficient accuracy by means of the slide rule.

In order to determine the validity of the assumption that  $\log .9 = 10 \log .99 = 100 \log .999$ , it is necessary to eliminate the error due to picking values of  $1 - .9^x$ ,  $1 - .99^x$ , and  $1 - .999^x$  from the curve. This was suggested to us by McCrady and was carried out, using accurate tables of  $1 - .9^x$ , etc. The calculations follow:

Fermentation Tube Results			Trial Values of $x$	Reduced Eq.		Probable No. B.Coli Per 100 C.C.	
				Exact McC.	Modified W. & W.	McC.	W. & W.
1/2	3/10	1/10.....	21 22	316 308	310	21	22
0/2	2/10	0/10.....	6 7	342 295	310	6	7
2/2	5/10	2/10.....	85 90	312 307	310	86	87
2/2	10/10	4/10.....	540 550	310.02 309.85	310	550	540
2/2	3/10	.....	35 40	306 293	300	37	37
2/2	1/10	.....	17 18	304 295	300	18	17
2/2	1/2	0/2.....	...	...	222	61	60
2/2	1/5	.....	...	...	250	27	28
2/2	3/5	2/5.....	...	...	255	140	138
Average .....						105	104

It is evident, from these figures, that the error due to the assumption under discussion is very small and in routine determinations is quite negligible. It is hoped, therefore, that the modification of McCrady's formula, because of the simplified operations involved in its solution and because of its accuracy within the usual practical limits, may lead to a wider use of the exact interpretation of fermentation-tube results in routine bacteriologic analyses.

# IMMUNE REACTIONS IN RABBITS INJECTED WITH MICROCOCCI FROM ACUTE POLIOMYELITIS\*

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In previous communications<sup>1</sup> one of us described a peculiar micrococcus obtained in cultures of the brain and spinal cord in acute poliomyelitis. The exact significance of the coccus has not been determined, but the results of cultures and experiments suggest that it may be of some importance. It has been found that opsonin apparently specific for this micrococcus occurs in the blood of persons recovering from poliomyelitis.<sup>2</sup> In order to arrive at a clearer understanding of the immunity reactions it seemed advisable to study the serum of rabbits, which were immunized against different strains of the coccus, with especial reference to the opsonins, complement fixing bodies, and agglutinins.

The bacteria were grown in ascites dextrose broth for 18 to 24 hours, then removed by centrifugation, washed, and suspended in sterile salt solution in sufficient amount to make a faintly cloudy, smooth suspension. Rabbits, 3 to 6 months old, were inoculated intravenously at 3 or 4 day intervals with increasing amounts, 0.25-2 c.c., of this suspension. After the rabbits had received several injections at short intervals and antibodies had accumulated in the blood, subcutaneous injections of 2 c.c. of the suspension were given weekly to maintain the antibody-content. Using similar methods, other rabbits were injected with strains of pneumococcus, of streptococcus viridans, of hemolytic streptococcus, and of *B. typhosus*, and their sera studied also. A moderate amount of antibody usually appeared after the 4th or 5th inoculation, and this increased decidedly after the 7th or 8th. When 0.02 c.c. of the rabbit serum gave complete inhibition of hemolysis with  $\frac{1}{120}$  of the anticomplementary unit of the corresponding antigen, the rabbit was then bled from the heart, the serum heated to 56° C. for 30 minutes, and stored in the refrigerator.

\* Received for publication May 2, 1917.

<sup>1</sup> Mathers: Jour. Am. Med. Assn., 1916, 67, p. 1019; Jour. Infect. Dis., 1917, 20, p. 113.

<sup>2</sup> Mathers and Tunnicliff: Am. Med. Assn., 1916, 67, p. 1935.

## COMPLEMENT FIXATION

Antisheep rabbit system was used in one-tenth the volume of the original Wassermann test. Two series of tests were made with each immune serum—varying the amount of antigen in one instance and the amount of serum in the other. As antigens similar suspensions of bacteria as for inoculation were used, with the exception that now they were heated to 56 C. for 30 minutes. The anticomplementary unit of each antigen was determined. In the tests in which the serum remained constant, the amount of antigen was varied from one-fourth to one one hundred and twentieth of the anticomplementary unit. When the serum was varied a constant amount of antigen representing one-fourth of the anticomplementary unit was used.

In the first experiments both heated and unheated serum was used, but the unheated serum was so frequently anticomplementary that its use was abandoned. When the antigen was varied, 0.02 c.c. of the serum was used. When the serum was varied, 0.02 c.c. was the first dilution and 9 subsequent dilutions were included. Fresh guinea-pig serum, 0.1 c.c. of a 1:10 dilution (2 units), was used as complement. Since it has been found that the serum of normal rabbits may give a positive Wassermann reaction, it seemed best to study the serum of each rabbit before inoculation. Accordingly several cubic centimeters of blood were drawn from the marginal ear vein of each rabbit and in every instance the serum was tested with the organism intended for antigen, with syphilitic liver extract and ascites dextrose broth. In no instance did the serum have any binding power. The Wassermann and ascites dextrose broth tests were repeated again after the animals were immunized; in no instance were positive results obtained.

Serum, antigen, and complement were incubated for 1 hour at 37 C.; then 0.1 c.c. of a 5% suspension of washed sheep corpuscles, and two units of antisheep amboceptor were added to each tube, the whole incubated at 37 C. for from 15 to 30 minutes according to the rapidity of the hemolysis in the controls. The customary controls (antigen, serum, and hemolytic), were carried through in each test.

So far observations have been made on the sera of 12 rabbits. Eight of these were injected with different strains of cocci from poliomyelitic material, one each with a pneumococcus, one with a hemolytic streptococcus, one with a *Streptococcus viridans*, and one with *B. typhosus*. The serum of a normal rabbit was included in each test as control. The results of the complement fixation tests using the two methods have been recorded in Tables 1 and 2.



TABLE 1.  
COMPLEMENT FIXATION WITH THE SERUM OF RABBITS IMMUNIZED WITH MICROCOCCI FROM POLIOMYELITIS

Serum of Rabbits, Injected with Organisms as follows:	Antigens											Controls					
	Tonsil, Polo- mye- litis	Polo- mye- litis 1	Polo- mye- litis 3	Polo- mye- litis 4	Polo- mye- litis 5	Polo- mye- litis 6	Polo- mye- litis 7	Polo- mye- litis 8	Polo- mye- litis 9	Polo- mye- litis 10	Polo- mye- litis 11	Pneu- mo- coccus	Hemo- lytic Strep- to- coccus	Strep- to- coccus Vir- dans	B. Typho- sus	Luetic Liver	Asetic Dex- trose Broth
Tonsil, poliomyelitis.....	+++	++	++	++	++	++	++	++	++	++	++	—	++	++	—	—	—
Poliomyelitis 7.....	++	—	++	++	++	++	++	++	++	++	++	—	++	++	—	—	—
Poliomyelitis 7.....	++	+++	++	++	++	++	++	++	++	++	++	+	+++	—	—	—	—
Poliomyelitis 10.....	+++	++	++	++	++	++	++	++	++	++	++	—	++	—	—	—	—
Poliomyelitis 6.....	+++	+	—	++	++	++	++	++	++	++	++	—	+	+	—	—	—
Poliomyelitis 8.....	+++	++	++	++	++	++	++	++	++	++	++	—	++	++	—	—	—
Poliomyelitis 11.....	+++	+++	++	++	++	++	++	++	++	++	++	—	+++	++	—	—	—
Poliomyelitis 1.....	+++	+++	++	++	++	++	++	++	++	++	++	—	—	—	—	—	—
Pneumococcus.....	++	++	++	++	++	++	++	++	++	++	++	+++	++	—	—	—	—
Hemolytic streptococcus	—	—	—	—	—	—	—	—	—	—	—	++	+++	+	—	—	—
Streptococcus viridans...	—	—	—	—	—	—	—	—	—	—	—	+	—	+++	—	—	—
B. typhosus.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Normal rabbit serum....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Controls	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

— = Complete hemolysis.  
 + = Inhibition of hemolysis in  $\frac{1}{4}$ – $\frac{1}{8}$  of anticomplementary unit.  
 ++ = Inhibition of hemolysis in  $\frac{1}{16}$ – $\frac{1}{32}$  of anticomplementary unit.  
 +++ = Inhibition of hemolysis in  $\frac{1}{32}$ – $\frac{1}{64}$  of anticomplementary unit.  
 ++++ = Inhibition of hemolysis in  $\frac{1}{64}$ – $\frac{1}{128}$ , etc., of anticomplementary unit.

TABLE 2.  
COMPLEMENT FIXATION WITH THE SERUM OF RABBITS IMMUNIZED WITH MICROCOCCI FROM POLIOMYELITIS

Serum of Rabbits, Injected with Organisms as follows:	Antigens											Controls						
	Tonsil, Polio- mye- litis	Polio- mye- litis 1	Polio- mye- litis 3	Polio- mye- litis 4	Polio- mye- litis 5	Polio- mye- litis 6	Polio- mye- litis 7	Polio- mye- litis 8	Polio- mye- litis 9	Polio- mye- litis 10	Polio- mye- litis 11	Pneu- mo- coccus	Hemo- lytic Strep- to- coccus	Strep- to- coccus Vir- dans	B. Typho- sus	Luetic Liver	Ascltic Dex- trose Broth	
Tonsil, poliomyelitis.....	.0001	.01	.0001	.0012	.00003	.00125	.0006	.0025	.0003	.0025	.01	.005	.01	.005	0	0	0	0
Poliomyelitis 7.....	.0003	.005	.005	.05	.01	.005	.0003	.00125	.00125	.0025	.005	.0025	.01	.01	0	0	0	0
Poliomyelitis 7.....	.0006	.005	.00125	.0003	.00003	.01	.00008	.0015	.0006	.0015	.02	.0025	.005	.01	0	0	0	0
Poliomyelitis 10.....	0	.005	.0003	.00125	.005	.0006	.01	.00125	.0012	.01	.005	.0025	0	0	0	0	0	0
Poliomyelitis 6.....	.0025	.01	.005	.005	.005	.0006	.005	.01	.0012	.01	.005	.005	.05	.01	0	0	0	0
Poliomyelitis 8.....	.005	.02	.0025	.0006	.005	.0006	.0025	.0025	.005	.0025	.005	.005	.005	.01	0	0	0	0
Poliomyelitis 11.....	.0003	0	.005	.005	.003	.0006	.005	.0025	.005	.005	.0025	.005	.005	0	0	0	0	0
Poliomyelitis 1.....	.0003	.0003	.005	0	0	.0006	.005	.00015	.00125	.005	.0003	.02	0	0	0	0	0	0
[Pneumococcus.....	0	.01	0	0	0	.005	0	0	0	0	.01	.0003	.01	0	0	0	0	0
[Hemolytic streptococcus	0	0	0	.0012	0	.0025	0	0	.005	0	0	.01	.0006	0	0	0	0	0
217.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B. typhosus.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
[Normal rabbit.....	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The figures in the table represent the lowest dilution of serum with which there was complete complement fixation.

The noteworthy points might be briefly reviewed. Antibodies for the homologous poliomyelitic coccus usually developed a short time before the group antibodies, but the general tendency of the complement-fixing substances was toward specificness for the group of poliomyelitis cocci. The serum of a rabbit injected with a poliomyelitic strain would bind complement with the majority of the poliomyelitis antigens but not with the control antigens, and vice versa. However, in some instances pneumococcus and streptococcus antigens gave positive reactions with the sera, but usually in lower dilutions than with the poliomyelitic antigens. Occasionally a serum with high fixing power with most of the strains would not contain any fixing substances for some one strain. Such discrepancies may be due to unknown physico-chemical conditions that are unavoidable. The Tables show that on the whole the group specificity is marked.

#### OPSONINS

The methods used were similar to those described in connection with the opsonic reaction in human cases.<sup>3</sup> Normal leukocytes were used and tests with serum of normal rabbits were made for control. The sera were diluted with salt solution in order to determine at what point the induced phagocytosis would exceed the spontaneous. Typical strains of poliomyelitic cocci were used. Cultures of *Streptococcus pyogenes*, of 4 different strains of *Streptococcus viridans*, and of a pneumococcus strain were also studied. The suspensions were made from 24-hour blood-agar-slant cultures, each suspension containing approximately the same number of organisms. The mixtures were incubated at 37 C. for 30 minutes, and the number of cocci taken up by 50 polymorphonuclear leukocytes determined. The serum was heated for 30 minutes at 56 C. before the tests were made.

In all instances in which complement fixation was marked, there was a high content of opsonin, the serum sometimes being active in dilutions as high as 1 : 7680. Moreover, these antibodies were specific for the poliomyelitis cocci, no change in reaction being obtained with the control bacteria. The results correspond to the previous observations on the specific opsonic reaction in the serum of persons recovering from poliomyelitis.

#### AGGLUTININS

Unheated serum was used in varying dilutions with the serum of normal rabbits for control. The various strains of poliomyelitic cocci

<sup>3</sup>Mathers and Tunnicliff: Jour. Am. Med. Assn., 1916, 67, p. 1935.

were grown on blood agar slants for 24-48 hours and suspended in enough sterile normal salt solution to make a faintly cloudy suspension. Equal amounts of this suspension and the various dilutions of serum were mixed, incubated for two hours at 37 C., and placed in a refrigerator for 24 hours, after which time the final readings were made. Cultures of a hemolytic streptococcus, a pneumococcus, and two strains of a *Streptococcus viridans* were tested also.

The results are of doubtful value because of the tendency of the poliomyelitis cocci to agglutinate spontaneously. It may be said, however, that specific agglutinins developed to a small degree in the serum of the immunized rabbits, not being definitely demonstrable in dilutions above 1:8 as a rule. In most instances the immune serum agglutinated all the poliomyelitic strains but not the control suspensions. In one case only did a serum agglutinate in a dilution as high as 1:128. Occasionally a serum active for one strain would not agglutinate other strains, but in no instance were the cocci agglutinated by the control sera any more than spontaneously.

#### SUMMARY

In the blood of rabbits injected with various strains of poliomyelitis cocci, specific agglutinins, opsonins and complement fixing bodies could be demonstrated. The complement fixing bodies were demonstrable in high dilutions and were specific for the poliomyelitis group in most instances. Occasionally, however, some fixation occurred with pneumococcus and hemolytic streptococcus antigens, but this was rare. Conversely, the pneumococcus immune serum and the hemolytic streptococcus immune serum gave some fixation with the poliomyelitic antigens; in the instance of the pneumococcus antigen the reaction seemed apparently nonspecific.

The opsonins were demonstrable in high dilutions and were specific—the serum being without opsonic effect on hemolytic streptococci, green-producing streptococci and pneumococci.

The agglutinins were demonstrable in low dilutions only. Spontaneous agglutination of the cocci interferes with agglutination tests.

These results suggest that serologic tests, especially opsonin determination, may prove of value in the diagnosis of acute poliomyelitis, in case the coccus is found to be associated closely with the disease.

# THE ETIOLOGIC AGENT AND THE LOCALIZING FACTOR OF THE ABSCESES IN MYOSITIS PURULENTA TROPICA \*

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Myositis purulenta tropica is a disease characterized by the development of deep muscle abscesses in the limbs or trunk, associated with rheumatoid pains and tenderness in the affected muscles and an irregular febrile temperature. The parts most often affected are the great muscles of the extremities, especially of the thigh and calf, and the muscles of the trunk, abdomen and chest. The muscles of the face, neck and hands are said to be exempt. There is a great tendency to multiple abscesses, several developing simultaneously or successive crops of them appearing at intervals. Owing to the deep location of the abscess in the muscle there is little or no superficial evidence of the inflammation, such as redness, heat and swelling; but by palpating deeply one can feel an induration with ill defined boundaries. Occasionally when the abscess is allowed to come to maturity a slight swelling of the surface is evident. The treatment is surgical; when opened and properly drained the abscess usually heals rapidly and completely. Unless acquainted with the disease one is liable not to find the pus pocket at operation from failure to go deep enough; as it frequently lies close to the bone. The quantity of pus is often large and may, on account of the pressure to which it is subjected in the depth of the great muscles, escape forcibly when the abscess is incised. Microscopically and culturally the pus usually shows pyogenic cocci, sometimes staphylococci, sometimes streptococci and sometimes both staphylococci and streptococci; occasionally the pus has been found sterile. Without surgical interference the abscess may work its way to the surface, or it may remain cryptic causing obscure symptoms and running a lingering course; in either case it is prejudicial to the general health, the patient becoming emaciated, falling into a decline and sometimes dying from a generalized infection.

The geographic distribution of myositis purulenta tropica is somewhat uncertain on account of other pathologic conditions apparently

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having been confused with it. The disease was first described by Ziemann<sup>1</sup> in West Tropical Africa. It has also been reported in Kaiser Wilhelm's Land,<sup>2</sup> South Sea Islands,<sup>3</sup> Panama Canal Zone,<sup>4</sup> and British Guiana.<sup>5</sup> The so-called mumu fever of Samoa has been considered identical with myositis purulenta tropica; but the symptomatology of the former disease, as described by Leber and Prowazek,<sup>6</sup> is that of an erysipelas-like edema of the skin and subcutaneous tissues. Of uncertain relationship also are the filarial abscesses described by Maxwell<sup>7</sup> in China. These abscesses occur in the scrotum, the intra-abdominal and intrathoracic regions, and in the limbs; but in the last location they are always in regions rich in lymphatic tissue and generally in the immediate neighborhood of the large vessels.

In the upper Madeira valley, Brazil, myositis purulenta tropica is of semi-frequent occurrence. During my stay of five months at Porto Velho 4 cases entered the hospital of the Madeiro Marmaré railway company. Of these cases one had 2 muscle abscesses, and two had suffered previous attacks of myositis. The location of the abscess was in the muscles of the thigh in two, of the calf in one, of the subcostal region in one, and of the chest in one case. The amount of pus removed from the abscesses at operation varied from 25 to 500 c.c. There was no history or clinical evidence of filarial infection in any of these cases. Microscopically the pus consisted of polymorphonuclear neutrophile leukocytes with no excess of eosinophiles. Microscopically and culturally *Staphylococcus aureus* was found in 3 and *Staphylococcus albus* in 1 of the cases. Thick blood smears made at night, and from two of the patients in the daytime, were negative for filarial larvae. Differential leukocyte counts of the blood of two of the patients did not show an eosinophilia.

The etiology of myositis purulenta tropica is generally considered to be filarial (Kulz,<sup>8</sup> Ziemann,<sup>9</sup> Wise and Minett<sup>5</sup>). Rodenwaldt<sup>10</sup> alone considers it a special form of pyemia. According to the conception of the filarial theory the adult female worm by her death or

<sup>1</sup> *Mediz. Berichte aus den deutschen Schutzgebieten*, 1904-5, 138.

<sup>2</sup> *Ibid.*, 173.

<sup>3</sup> *Arch. f. Schiffs- u. Trop.-Hyg.*, 1912, 16, Supp. 4.

<sup>4</sup> Personal communication.

<sup>5</sup> Report of the Advisory Committee for the Tropical Disease Research Fund for the years 1912-13, pp. 108-114.

<sup>6</sup> *Arch. f. Schiffs- u. Trop.-Hyg.*, 1911, 15, pp. 409-430.

<sup>7</sup> *Brit. Med. Jour.*, 1901, 2, p. 609.

<sup>8</sup> *Arch. f. Schiffs- u. Trop.-Hyg.*, 1912, 16, p. 313.

<sup>9</sup> *Ibid.*, 1913, 17, p. 469.

<sup>10</sup> *Ibid.*, 1914, 18, p. 41.

through the deposition of ova, or perhaps also by the production of toxins in the lymphatics, incite the tissue reactions that result in the development of the abscess. The pyogenic cocci frequently found in the pus are considered to be secondary invaders. Kulz<sup>8</sup> goes farther and ascribes the etiology of this disease to a certain species of filaria, *Loa* (*Filaria*) *loa*. Ziemann<sup>9</sup> also considers *Loa loa* to be of especial etiologic importance in myositis, but thinks that *Filaria bancrofti* may also be considered in its production.

In considering the etiology of myositis purulenta tropica one must distinguish between the etiologic agent which incites the pus formation and the localizing factor which determines the peculiar situation of the abscesses in the muscles. These two factors, although hitherto confused, are quite distinct. As an illustration of this distinction, osteomyelitis, and endocarditis may both be caused by infections with *Staphylococcus aureus*, but the localizing factor which determines the situation of the infection and consequent lesions in the two diseases is evidently quite distinct from casual agent. The etiologic agent of myositis purulenta tropica is evidently not filariae but the pyogenic cocci, because:

1. The pyogenic cocci, as all authors agree, are usually found in the pus, and they are probably always present in the early stages of the abscess. The occasional absence of these organisms reported by Kulz,<sup>8</sup> Ziemann,<sup>9</sup> and Wise and Minett<sup>5</sup> are probably due to the age of the abscess. It is well known that the pyogenic cocci frequently disappear in the later stages of suppurative processes.

2. The pyogenic cocci found in these deep muscle abscesses cannot be secondary invaders in the ordinary sense because they can reach these locations only through the blood stream.

3. The cellular reaction in these muscle abscesses is not characteristic of filarial infections. The cellular reactions to living filariae are fibrous tissue and eosinophiles (Bahr<sup>11</sup>), and to dead filaria calcification (Wise,<sup>12</sup> Bahr<sup>11</sup>); while the cellular reaction in myositis purulenta tropica is polymorphonuclear neutrophiles, which is the characteristic reaction to the pyogenic cocci.

4. From the prevalence of filarial infections in certain regions and the frequency with which dead and calcified filariae are found in the tissues at autopsy, it is evident that filariae of themselves are incapable

<sup>11</sup> Jour. London School of Trop. Med., Supp. 1, 1912.

<sup>12</sup> Jour. Trop. Med. and Hyg., 1910, 13, p. 137.

of producing abscesses. On the other hand, the pyogenic cocci are capable of producing, and if localized in the muscles must inevitably produce, the total clinical and pathologic picture of myositis purulenta tropica.

It is generally recognized that a pyemia always depends on an antecedent septicemia, which may be of only temporary character. It is probable that in the majority of pyemias the invasion of the blood is indirect as a result of the involvement of the vessels in the primary inflammatory focus and the consequent formation of thrombi. From these infected thrombi small particles containing bacteria pass into the general circulation and reach capillaries too small to allow their passage, or because of pressure on or injury to the capillaries become fixed in certain locations where they multiply. The primary lesion, which serves as a portal of entry of the bacteria, may sometimes be evident but is often obscure, as in slight skin lesions which may be overlooked or healed before the pyemia develops. Roenwald<sup>10</sup> believes that in myositis purulenta tropica in Togo the portal of entry of the pyogenic cocci is through the lesions produced by sand fleas or jiggers (*Dermatophilus penetrans*) which are very prevalent in that region.

It is not, however, the portal of entry of the pyogenic cocci — for which there is abundant opportunity in native peoples of tropical countries — but the localizing factor which makes myositis purulenta tropica a pyemia sui generis. Pyemias occur everywhere, but localization in the deep muscles is limited to the tropics and apparently to certain regions of the tropics; consequently it would seem that there must be some localizing factor peculiar to these regions. It is in this connection that the filariae may come into consideration. *Loa loa*, notwithstanding the arguments of Kulz,<sup>8</sup> can be excluded both by its geographic distribution and by the habitat of the adult worm in the subcutaneous connective tissue. *Filaria bancrofti* is more open to suspicion as a localizing factor. Wise and Minett<sup>5</sup> have supplied the best evidence incriminating this worm. These authors made a careful search of the contents of 28 deep seated abscesses in British Guiana and found adult *Filaria bancrofti* or pieces of them in 22 of the cases. It is difficult, however, to understand why myositis purulenta tropica is apparently absent in many countries, such as Arabia, India, Indo-China, the Philippine Islands, and Australia, where infections with *Filaria bancrofti* are common. In the Madeira valley, according to the records of the Candelaria Hospital, clinical evidence of filariasis is rarely if ever met with. While time did not permit an extended

search for such infections by blood examinations, a considerable number of thin and thick blood smears were examined for malarial parasites without encountering filarial larvae. None of my cases of myositis purulenta tropica showed any clinical evidence of filariasis. The adult *Filaria bancrofti* lives in the lymphatics and might by the obstruction of the lymphatic vessels cause pressure or injury to the neighboring capillaries and thus cause a localization of the pyemic foci. If this be true one might expect that the localization would occur most frequently, as do the other manifestations of filariasis, in the regions of the body richest in lymphatics instead of the deep muscles where the lymphatic vessels are fewer in number and smaller. It may be, however, that the smallness of the lymphatic vessels in the deep muscles is an important factor in the process. Such small lymphatic vessels might be more liable to become obstructed by the adult filariae, and cause pressure or injury to the adjacent blood capillaries, than the larger lymphatic vessels of the subcutaneous tissues.

From the foregoing it may be concluded that myositis purulenta tropica is a peculiar form of pyemia. The causal agent in its production is most certainly the pyogenic cocci usually found, and probably always present at some time, in the abscesses. The localizing factor which determines the peculiar situation of the pyemic foci is probably filarial, but the evidence on this point is not conclusive.

## A STREPTOBACILLUS FROM URINE\*

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In the routine bacteriologic examination of specimens of catheterized urine we have repeatedly isolated an organism which we were unable to identify with any of the bacteria described in the literature available. As it occurs in the urine, the organism bears a striking morphologic resemblance to a long chain of streptococcus. In early subcultures on solid media it is coccoid, but later becomes definitely bacillary in form. In fluid media there is constant chain formation. We believe therefore that this organism should be included among the streptobacilli.

Attention is called to this streptobacillus on account of the readiness with which it may be confused with the streptococcus. At first regarded as a contamination, it was not until its association with bladder symptoms was noted, that a more extensive study of its cultural and biologic characteristics was undertaken.

### MORPHOLOGY AND CULTURAL CHARACTERISTICS

Only those characteristics are recorded that are essential to the identification of the organism.

It is a gram-positive, pleomorphic bacillus.

In recent cultures the individuals are mostly coccoid, with sharp pointed ends, occurring frequently in pairs resembling pneumococci. In older cultures the predominant form is a short, rather plump bacillus. In broth cultures the organism occurs in long and short chains, or as diplococci. No capsule demonstrable.

On agar there is a moist, gray growth, confined to streak, sometimes sticky in consistency.

On glucose (2%) ascitic fluid agar growth the same as on agar; marked precipitation<sup>1</sup> in twenty-four hours.

On glucose (2%) broth a diffuse clouding, heavy, granular sediment.

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<sup>1</sup> Libman: Jour. Med. Research, 1901, 1, p. 84; Bull. Johns Hopkins Hospital, 1906, 17, p. 215.



On human blood agar a gray, moist growth, no hemolysis.

Inulin is not acidified.

Litmus milk is acidified and coagulated in four days.

Gelatin is not fluidified.

Bile does not dissolve the organism.

Potato, a heavy white, moist growth.

Acid is produced in dextrose, sucrose, lactose; none in mannite; no gas.

This communication is based on the study of 3 strains isolated from the following cases:

Organism A was recovered from a case of chronic prostatitis with dysuria and frequent micturition. The organism has been recovered at intervals over a period of 6 months, always in association with *B. pyocaneus*. Slight improvement under local treatment and autogenous vaccines.

Organism 332 was recovered from a case of mixed tumor of the right kidney with dysuria, hematuria, and pain on the right side of abdomen. Right nephrectomy; recovery.

Organism 464 was recovered from a case of chronic prostatitis and edema bulbosum vesicalis with dysuria, dribbling of urine, and pain in the region of the bladder. Symptoms were relieved by daily irrigations with nitrate of silver.

It appears to us that this streptobacillus has a special predilection for invading the urinary tract and maintaining itself there. We have no information as to its portal of entry or as to whether it reaches the bladder by an hematogenous, an ascending or a descending route.

To determine the pathogenicity of the organism for animals, several mice and guinea-pigs were injected intraperitoneally and subcutaneously with a 24-hour culture of Organism A. One mouse died 11 days after an intraperitoneal injection. The findings were negative and *B. proteus* alone was recovered from the peritoneal cavity. The other animals remained well. A rabbit was injected intravenously with the same culture. It remained well for 5 days and was then re-injected intravenously. The animal showed no signs of illness and was killed 6 days after the second injection. Cultures from the heart blood remained sterile but from the urine the injected organism was recovered in pure culture. We then made a series of experiments to test whether the organism would consistently show predilection for the urinary tract of rabbits.

A series of preliminary experiments was made to determine the sterility of rabbit urine as well as the best method of securing specimens of urine from the same rabbit at repeated intervals.

It was first attempted to express the urine from the rabbit's bladder directly into a sterile vessel. This did not prove satisfactory, however, as a number of contaminating bacteria were usually found, including cocci, which proved difficult to differentiate from the streptobacillus.

Under anesthesia, the hair was removed from the lower abdomen with a solution of sodium sulphid, and the skin was sterilized with tincture of iodine. Under aseptic precautions, an incision, about 1 cm. long, was made above the symphysis, the bladder grasped with anatomic forceps and delivered. The bladder was then punctured with a fine needle and the urine aspirated. The wound was closed with silk sutures. The procedure takes from 3 to 5 minutes. Healing was practically always by primary union, and in the few cases where slight infection of the skin wound occurred the animal was not again aspirated until the infection had entirely disappeared.

The bladders of 6 rabbits subsequently used in our experiments were aspirated in this manner, and the urine obtained from all was sterile.

In view of the uniformity of these results it seemed unnecessary to make preliminary cultures in the remaining animals of the series.

The series of experiments is summarized as follows:

SUMMARY OF EXPERIMENTS

Organism	No. of Animals	No. of Urine Cultures Positive	No. of Ureter Cultures Positive
A	12	11	2
332	4	4	
464	4	2	1

It is evident that the streptobacillus, even in enormous intravenous doses, is not lethal for rabbits. Blood cultures taken from the ear vein show that the organism may remain in the circulating blood for 24 hours after intravenous injection. This observation was made repeatedly. In only one instance was the blood culture positive after 48 hours, then becoming negative.

The organism has been recovered from the urine in the bladder as early as 24 hours after the intravenous inoculation and quite regularly after 48 to 72 hours. It has been recovered from the urine by aspirations of the bladder at intervals over as long a period as 40 days.

In 3 animals, whose urine contained the streptobacillus, a cannula was inserted into the ureter about 1 cm. below the pelvis of the kidney, and the organism recovered in large numbers in pure culture from the urine thus collected.

Cultures were made from the emulsified kidneys of two of these animals and no streptobacilli were recovered. The cultures from the kidneys of another animal whose ureters were not catheterized were positive. It is possible that the result in this animal was due to inclusion in the emulsified tissue of a portion of the pelvis containing streptobacilli.

The kidneys from 6 of the rabbits were carefully examined. None showed any gross or microscopic lesions which could be attributed to the organism in question, although several showed the so-called idiopathic or spontaneous nephritis commonly found in rabbits.

Those kidneys which were obtained a considerable time after injection of the streptobacillus showed no bacteria in sections stained with the Gram-Weigert method. These sections were prepared from alcohol-fixed material by the paraffin method, and the stain used was filtered through a Berkefeld filter. Two kidneys, however, removed from animals only a few days after the injection showed typical chains of the organism lying on the pelvic mucous membrane, while in one kidney a small number of organisms in clumps were seen in the loops of a few of the glomerular tufts.

The aspirated urine from several animals contained a trace of albumin and epithelial cells which are frequently normal constituents of rabbit urine.

It seems reasonable to suppose that the streptobacillus passes from the blood through the kidneys into the urine. We have not discovered just how this occurs. We believe that the weight of evidence indicates 1, that the kidney does not normally filter bacteria from the blood into the urine; and 2, that bacteria only pass from the blood through the kidney into the urine when some lesion is present, even though this may not be discoverable with the microscope.

Nephritis is so common in adult rabbits that practically none are absolutely free from it. It is probable that the organism gained passage to the urine through these abnormal areas. On the other hand, in another series of experiments on rabbits, we have injected intravenously large doses of an hemolytic streptococci recovered from gastric ulcers. When these animals were sacrificed cultures were taken from the heart blood, bile, and urine. In a few animals the blood contained

streptococci, but in only one of these were streptococci found in the urine. The urine from the other animals was sterile. Similarly one of us has injected *Staphylococcus aureus* into the renal artery of dogs and collected the urine for 2 hours from both ureters. The staphylococci were not found in the urine from either the injected or uninjected side although both the general and splanchnic circulation were teeming with these organisms.

It would seem, therefore, that this streptobacillus possesses some peculiar biologic property which enables it to live in the urine and maintain itself in the bladder and probably on the mucous membrane of the ureter and renal pelvis also.

#### CONCLUSIONS

A hitherto undescribed, gram-positive streptobacillus has been isolated repeatedly from the urine of cases suffering from bladder symptoms.

It is of low virulence and large doses injected intravenously into rabbits do not kill the animals. The organism does not usually remain in the circulating blood longer than 24 hours.

After intravenous injection the streptobacillus can be recovered from the bladder urine sometimes as early as 24 hours, and regularly after 48 to 72 hours.

It has also been recovered from the urine secured by ureteral catheterization several days after intravenous injection, but has not been recovered with the same regularity from the kidneys of these animals.

This organism seems, therefore, to possess peculiar biologic properties which enable it to maintain itself in the urinary bladder, and probably in the ureter and renal pelvis as well, for an indefinite period.

# OBSERVATIONS ON THE GROWTH OF STREPTOCOCCI IN BLOOD-CARBOHYDRATE MEDIUM \*

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Carbohydrates not only serve as an important food substance for many bacteria but are most useful for the identification and differentiation of closely related varieties. Blood and blood serum in recent years have come into use quite extensively as an aid in differentiation of organisms. In addition to other means the ability of germs to take the corpuscles has been taken advantage of, especially in connection with the examination of cocci. The interaction, therefore, of blood and carbohydrates in mediums under the influence of bacterial growth is of significance both practically and theoretically.

Since Schottmüller called attention to the hemolytic action of streptococci on blood and on this basis divided them into 3 types: the hemolyticus, the viridans, and the mucosus, this method has in many laboratories come into general use for isolating and differentiating this group. For this purpose it has proved an invaluable aid. By many workers, especially in this country for the past 15 years, the method has been tested out quite thoroughly and its limitations and possibilities have been fairly well determined. I may refer in this regard to the early work in this country of Weaver, Ruediger, and Rosenow, to the more recent work of Smith and Brown,<sup>1</sup> and to the classifications of streptococci advocated by Holman<sup>2</sup> and by Lyell;<sup>3</sup> also to the recent work of Becker.<sup>4</sup> The hemolytic property, like many other properties of bacteria, is not absolutely constant but ordinarily under uniform conditions is sufficiently so to be of real value in differentiation.

It has long been known that sugar in mediums interfered with the production of characteristic zones of hemolysis on plate cultures.

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<sup>1</sup> Jour. Med. Research, 1914, 31, p. 455.

<sup>2</sup> Ibid., 1916, 34, p. 377.

<sup>3</sup> Ibid., 1914, 30, p. 487.

<sup>4</sup> Jour. Infect. Dis., 1916, 19, p. 754.



It was pointed out by Ruediger<sup>5</sup> that in glucose blood mediums hemolytic streptococci failed to produce a clear zone, but became surrounded by an indefinite greenish or brownish zone. He showed also that lactic acid when dropped on a blood-agar plate caused a similar greenish zone to form about itself. Cole<sup>6</sup> has demonstrated that pneumococci change oxyhemoglobin to methhemoglobin in artificial medium, and that the greenish or greenish-brown color about pneumococcus colonies on blood plates is due to this phenomenon. Blake<sup>7</sup> has made the same observation with streptococcus viridans. Sugars very appreciably accelerate this phenomenon.

Hiss<sup>8</sup> called attention to the value of inulin serum water mediums in the differentiation of pneumococci and streptococci. As a result of the fermentation of inulin by pneumococci, there resulted from the acids formed a precipitation and coagulation of the albuminous substances in the medium, causing it to become white and solid. This change does not occur in such medium inoculated with streptococci. This method has been modified by Ruediger,<sup>9</sup> who used a higher proportion of serum or ascites fluid in order to facilitate the growth of certain strains of pneumococci, which did not grow well in Hiss serum water medium.

Blood mediums should be standardized more carefully than they have been in the past. Attention has been called to this by nearly everyone who has worked on the subject. Uniform methods should be used so that the results of various workers may be comparable.

Blood, when used in mediums, should be reasonably fresh. Standing in a cool chamber for several days, however, does not impair its value to any marked degree. There are certain appreciable differences between human blood and the blood of various animals as shown by Becker.<sup>4</sup> One-half c.c. of defibrinated human blood added to 5 c.c. of melted agar cooled to approximately 42 C. is the proportion of blood used in the experiments herein reported.

With a series of strains of streptococci, I have made observations on the effect of their growth on blood mediums containing a variety of sugars both fermentable and nonfermentable; 1% sugar mediums were first used, but on account of an occasional indefinite or delayed reaction the amount of sugar was increased to 2 or 3% in the mediums and either one of these amounts was used in the later experiments. I should recommend 2% for routine work.

It was noted that when streptococci are grown on fermentable sugar blood mediums, the mediums acquire a brown turbidity, beginning at first near the colonies, and later involving the entire plate or tube. This change may be attributed to the action of acids produced

<sup>5</sup> Jour. Infect. Dis., 1906, 3, p. 663.

<sup>6</sup> Jour. Exper. Med., 1914, 20, p. 363.

<sup>7</sup> Ibid., 1916, 24, p. 315.

<sup>8</sup> Ibid., 1902, 10, p. 317.

<sup>9</sup> Jour. Am. Med. Assn., 1906, 47, p. 1171.

by the bacteria, and in order to analyze this point further systematic tests have been made of the affects of the growth of a number of strains of streptococci on mediums containing the various sugars. The results are given in Table 1.

This table shows the fermentation of sugars as determined by titration, and it also indicates by the plus sign those blood-sugar plates and tubes which developed the striking brown turbidity. It will be seen that the reaction as manifested by the development of brown turbidity corresponds in every instance to the positive fermentation tests. With those sugars which are not fermented by the streptococci the blood mediums remain red, and the typical clear zones of hemolysis on plate cultures are quite like those produced on plain blood-agar plates.

TABLE 1  
EFFECT OF STREPTOCOCCI WHEN GROWN ON CARBOHYDRATE (2%) BLOOD AGAR PLATES AND TUBES

Number of Strain	Glucose		Lactose		Maltose		Sallein	
	Blood Reaction	Titra- tion	Blood Reaction	Titra- tion	Blood Reaction	Titra- tion	Blood Reaction	Titra- tion
72	+	6.05	+	4.37	+	5.05	+	6.05
183	+	5.15	+	5.05	+	5.05	+	5.05
300	+	6.25	+	4.55	+	5.55	+	5.05
228	+	6.35	+	2.71	+	5.25	+	4.71
140	+	5.87	+	4.55	+	5.05	+	4.85
290	+	5.05	+	4.65	+	3.35	+	5.05
41	+	6.55	+	3.65	+	5.25	+	4.05
134	+	6.37	+	4.55	+	4.05	+	4.55
310	+	6.05	+	4.87	+	5.05	+	5.35
6	+	6.10	+	4.60	+	5.30	+	5.10
211	+	4.55	+	3.65	+	3.85	+	4.35

These reactions are so clear cut that sugar blood tubes may be used for determining fermentation properties instead of litmus mediums. By adding a small amount of lactic acid to mediums, either in plate or tube, the change that occurs is quite indistinguishable from that caused by the growth of the acid-producing bacteria.

The zone of hemolysis on the fermentable sugar-blood plates was often indistinct, and at times not visible at all. Sometimes the zone at first was fairly clear, later becoming more turbid, but not persisting as a clear and definite zone, like that which hemolytic streptococci produce on the plain blood mediums.

This reaction is entirely comparable to the reaction observed in the sheep serum inulin mediums devised by Hiss for the differentiation of streptococci and pneumococci. When the defibrinated blood is used we get not only a precipitation of the serum constituents, but also a brownish discoloration of the red blood cells, due to acids and no doubt other changes which make the differentiation decidedly

striking. Since blood and carbohydrates are no doubt coming more and more into use in growing and determining bacteria, I suggest this method as of importance in the study and classification of members of the pneumo-streptococcus group.

An attempt was made to test the relation of hemolysis to acid-production by streptococci. An experiment was designed to neutralize the effect of the acid by the addition of powdered  $\text{CaCO}_3$  to the blood-sugar mediums.

Glucose and lactose were used. On such mediums when freshly made and plated the particles of  $\text{CaCO}_3$  are seen evenly distributed. After incubation the  $\text{CaCO}_3$  particles around the streptococcus colonies are dissolved, to some extent in 24 hours, much more so in 48 or more hours. The zone of solution of  $\text{CaCO}_3$  particles is larger than the zone of hemolysis. For 24-48 hours

TABLE 1.—Continued

EFFECT OF STREPTOCOCCI WHEN GROWN ON CARBOHYDRATE (2%) BLOOD AGAR PLATES AND TUBES

Saccharose		Mannite		Raffinose		Inulin	
Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration
+	Acid	+	3.35	0	1	0	0.95
+	Acid	0	1.00	0	1	0	0.95
0	1.5	0	1.00	0	1.1	0	1.25
+	4.65	0	1.5	0	1.5	0	1
+	3.55	+	3.05	0	1	0	0.91
+	5.05	0	0.95	0	0.83	0	1.2
+	4.25	0	0.95	0	1	0	1
+	4.6	0	0.98	0	0.85	0	1.08
+	5.55	0	0.9	0	1.2	0	0.91
+	4.70	0	1	0	0.9	0	1.08
+	3.55	0	0.9	0	1	0	1.08

the blood on the  $\text{CaCO}_3$  plates does not change materially; the carbonate seems to be appreciably protective for the corpuscles for a time at least. Later the plates slowly turn brownish and turbid, but usually not to the same extent as in the plates without  $\text{CaCO}_3$ . The colonies on the  $\text{CaCO}_3$  plates become somewhat larger than when grown on mediums without  $\text{CaCO}_3$ .

Control experiments were made to test the effect of  $\text{CaCO}_3$  on plain blood-agar plates and the streptococcus hemolytic zones. The plain blood agar containing  $\text{CaCO}_3$  remains red and appears fresh for a week or more, at room or incubator temperature, and shows no difference from a control plate made without  $\text{CaCO}_3$ . On  $\text{CaCO}_3$  blood plain agar plates sown with hemolytic streptococci, the zones are about normal in size and appearance and remain so for a week or longer. About the colonies on such plates the particles of  $\text{CaCO}_3$  remain indefinitely. Therefore the zone of hemolysis is not affected by  $\text{CaCO}_3$ , and it would seem further that it bears no relation to acid-production in the case of typical hemolytic streptococci. Twenty strains were tested and all reacted alike.

The question as to whether sugars inhibit hemolysis was inquired into, using the blood-agar plate. Six typical hemolytic streptococci were tested first on plain blood plates to determine the diameter of the zone of hemolysis. This was found to vary 2-4 mm. for the various strains. They were also

plated on  $\text{CaCO}_3$  blood-glucose agar, and the size of the zones were noted and compared with the controls. The average size of the zones of many colonies of each of the strains revealed no essential differences. It was noted here again that the  $\text{CaCO}_3$  particles in the colony, and for some distance about it, were dissolved in the sugar mediums. The clearing zone of the  $\text{CaCO}_3$  particles is decidedly larger than that of the blood corpuscles. This is another point indicating that the zone of hemolysis is not dependent on acid-production. That acid- and hemolysin-production go on together was shown by these experiments, but it was noted that apparently the rate of the acid-production is relatively slower than that of hemolysis. At any rate the blood corpuscles seem more sensitive to hemolysin than do the particles of  $\text{CaCO}_3$  to the acid in the concentration in which these substances exist about the colonies.

It would appear that the acid is decidedly more diffusible in the mediums than is the hemolysin. The hemolysin diffuses into the medium rather rapidly during the first 24 hours, then slowly as judged from the size of the zone. Furthermore, the margin, in most strains of streptococci but not in all, is very clear cut and definite. On the sugar plates the acid diffuses rather rapidly, and continues to diffuse for several days so that, as a rule, sooner or later the entire plate becomes turbid and brown, even though few colonies may be found on it. The margin of the acid zone also is not clear and definite as is usually the margin of the zone of hemolysis.

Since mediums are commonly made with meat or extract which contains some muscle sugar, the point whether or not such sugar has any appreciable effect on plate hemolysis was inquired into. Eight typical hemolyzing strains of streptococci were selected for the tests. Plain agar was prepared, using meat; also the same medium, using ordinary meat extract. As controls sugar-free agar was used. On all these mediums no differences were noted between the character of the hemolytic zones about the streptococci. It would seem that the small amount of sugar in such mediums is too small to appreciably affect the nature or intensity of hemolysis.

Attention is called to the fact that certain peptones, on the market especially since the war, contain considerable quantities of sugar, and when such are used in the preparation of mediums, they are not suitable for the differentiation of bacteria by blood-agar methods. When Witte's peptone is not available the peptone used should be investigated as to its sugar content. This point is especially important in the preparation of standard medium and particularly of sugar-free medium. I would point out also that certain peptones darken the medium to such an extent that clear cut blood reactions are not readily obtained.



The amount of NaCl in medium is important in relation to hemolysis. When no salt is added, as is well known, blood introduced into such mediums immediately hemolyzes. Plain agar was prepared containing amounts of NaCl, 0.1% -1.5%. Ten strains of hemolytic streptococci, 5 virulent and 5 nonvirulent, were tested on this medium to which one-third c.c. of human defibrinated blood to 5 c.c. was added before plating. The fragility of the corpuscles in this medium is a little below the resistance in pure salt solution, as might be expected. At 0.4%, at which in pure salt solution there is slight hemolysis, there was apparently no hemolysis as observed both with the microscope and the naked eye. Above this point the zones of hemolysis were normal in the various concentrations tested. If to such salt-free medium or medium with low NaCl content, sugar, either fermentable or nonfermentable, be added in the usual concentration of 1% or in any sufficient concentration, hemolysis will not take place, because the sugar protects the corpuscles. For such medium, therefore, salt is not a necessary constituent for the prevention of hemolysis.

#### SUMMARY

Streptococci when grown on a fermentable sugar-blood medium cause a characteristic brownish turbidity which soon involves the entire tube or plate.

On nonfermentable sugar-blood medium no such change is noticed, hemolysis occurring as on plain blood mediums.

This change is sufficiently definite and constant to be of value in determining carbohydrate reactions.

It is useful in routine work since practically all varieties of streptococci grow well in this medium.

Powdered  $\text{CaCO}_3$  added to sugar-blood agar will protect the medium for a time against the acids formed, but not permanently.

The carbonate added to plain blood medium has no appreciable effect on hemolysis by streptococci.

The presence of sugar in various peptones, at present on the market, is a disturbing factor in the blood-plate culture of bacteria.



# FURTHER OBSERVATIONS ON SUBCUTANEOUS ABSCESSSES IN RABBITS

## THE CARRIER STATE AND ITS RELATION TO RABBIT SEPTICEMIA \*

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In a previous paper<sup>1</sup> I called attention to the not infrequent occurrence of well defined subcutaneous abscesses in rabbits. From these I was able to obtain often in pure culture a small gram-negative, non-motile, coccoid bacillus with some tendency to form chains, which had some of the properties of *B. influenzae*, but in certain respects was distinctly different. This organism was isolated and grown on artificial mediums, and on subcutaneous injection into healthy rabbits it reproduced typical abscesses from which it again could be readily isolated. An identical organism was isolated from rabbits suffering or dying from respiratory infections, which not infrequently attacked in epidemic form the animals in the laboratory. These epidemics are commonly observed and are called by a variety of names, Snuffles, rabbit influenza, 'Brustseuche,' rabbit septicemia, etc. Many such epidemics, if not all, are due to bacilli of this type, organisms which are classed in the hemorrhagic-septicemia group and are closely related to or probably identical<sup>2</sup> with such organisms as *B. bovisepiticus*, *B. suisepiticus*, *B. avisepiticus*, etc.

Many workers have described bacteria of this general type as the causative agents in epidemics in rabbits, but there exists considerable disagreement in their descriptions of the bacilli. Furthermore, the clinical description of these outbreaks by various observers differs to some degree so that one can readily understand why they are usually considered as a group rather than a single variety. Laven,<sup>3</sup> for example, has tabulated the various bacteria that have been found as apparently causative organisms in epidemics in rabbits and guinea-

\* Received for publication June 6, 1917.

<sup>1</sup> Jour. Infect. Dis., 1913, 12, p. 42.

<sup>2</sup> Besemer: Jour. Bact., 1917, 2, p. 77.

<sup>3</sup> Centralbl. f. Bakteriöl., I, O., 1910, 54, p. 97.

pigs. Fourteen gram-negative bacteria have been described, all of which differ from each other in some detail. All, however, when injected into animals produce similar pathologic lesions.

However, since the work of Hüppe<sup>4</sup> the bacillus of rabbit snuffles or rabbit septicemia has come to be a definitely recognized bacillus belonging to the hemorrhagic-septicemia group, and these various organisms, or at least most of them, no doubt are strains belonging to this group, having acquired varying degrees of virulence and specific selective action for various animals,<sup>5</sup> and no doubt certain other modified characteristics.

Schimmelbusch and Mühsam<sup>6</sup> described a small organism isolated from abscesses in rabbits which agrees in essential details with this bacillus. No doubt they were dealing with the same organism but did not recognize that it belonged to the hemorrhagic-septicemia group of Hüppe. This bacillus is usually referred to in the literature independently<sup>7</sup>, and so far as I can determine its relation to the hemorrhagic-septicemia bacillus has not been recognized heretofore. This is the only reference I have found of examinations made on these subcutaneous abscesses, and though the authors in this report considered the bacillus a specific causative organism, there is no doubt from their description that it belongs to the septicemia group.

Since my first article, which included data from 18 animals, I have observed and examined 12 or more similar abscesses in rabbits from which I have always been able to isolate the same small bacillus. The morphology of these strains agrees with that given in the first series that I studied. A point in their morphology that was brought out previously but may be again noted is the tendency of the bacillus, especially of certain strains, to produce thread forms quite like the influenza bacillus.

The organism yields its rather delicate, moist, somewhat slimy growth on blood agar or other mediums containing animal fluids for a few generations. After about the 3rd or 4th generation the growth may be very scant or fail completely even in rich blood agar. If the oxygen supply is limited by growing in a tube connected by rubber tubing with a 2nd tube inoculated with the hay bacillus, the growth is not decidedly enhanced.

<sup>4</sup> Berl. klin. Wehnschr., 1886, 23, p. 753.

<sup>5</sup> Hutyrá: Cited by Kolle and Wassermann, Handb. d. path. Mikroorg., Ed. 2, 1913, 6, p. 81.

<sup>6</sup> Arch. f. klin. Chir., 1896, 52, p. 564.

<sup>7</sup> Cited by Kolle and Wassermann, Handb. d. path. Mikroorg., 1913, 6, p. 181.

I have tested some of the strains recently isolated on the various sugars, as was done previously with other strains, and found that they agree in every detail. That is, they produce acid without gas-production in dextrose, saccharose, and mannite, but not in inulin, raffinose, salicin, maltose, or lactose. These reactions agree in every way, so far as they are comparable, with recent data given by Besemer<sup>2</sup> on the hemorrhagic-septicemia group.

The virulence of the infections varies considerably. I have observed epidemics in which the majority of the animals attacked died in a few days. On the other hand, the infection may be relatively mild and manifest itself by only a slight purulent discharge from the nose, or even by a trace of moisture about the nostrils. The variation in the virulence of the strains of this organism was readily demonstrated experimentally.

Two rabbits were injected intranasally with 1 c.c. of a broth culture of a strain isolated 3 weeks previously from the nose of a sick animal. One remained entirely well and the other developed a very slight discharge from the nose on the 2nd day. One week later both were again inoculated with 1 c.c. intranasally with another strain just isolated from the lung of an animal dead of the disease. One died in 2 days of general septicemia, and the other died in 3 days with typical fibrinous pericarditis, pleuritis, and peritonitis, and acute pneumonia and septicemia. Pure cultures of the bacilli were isolated from the blood and various organs.

Not all rabbits are equally susceptible. One animal resisted intranasal inoculation of 2 different strains which readily killed other rabbits; however on subcutaneous injection of 0.25 c.c. of 1 of these same strains, it died of septicemia in 48 hours.

Occasional outbreaks in the laboratory animals and the fact that the disease is practically endemic now in many laboratories and animal-breeding houses led me to note certain points in connection with carriers in this disease, a point which has not received much attention. Of the animals that do not die of the infection, a considerable number improve and the disease assumes a chronic form, in which state perhaps only a slight discharge or moisture about the nose continues.

I have had 5 animals under observation for many months. They were at first quite ill, but later improved. From the slight nasal discharge of these animals the small bacilli have been cultivated repeatedly during this period. One animal has now been under observation for about 11 months; the others 4-9 months. They are in good general condition, are not emaciated, and might easily pass for normal animals. One of them, which had been under observation for 4 months, developed a disease of the middle and internal ear, which caused him to rotate his head to the left. He was killed and there was found an otitis

media with extensive abscess-formation containing thick grayish pus, which yielded a pure growth of the small bacillus. The mucosa of the nasal cavities was slightly inflamed, there being a slight nasal discharge.

Subcutaneous abscesses have not been observed in such animals unless the bacteria are injected under the skin, where they may form and remain for a long time. Such animals, therefore, are not immune to the bacilli even to their own strains. Autogenous organisms from the nose were injected subcutaneously into 3 animals with positive results, the animals dying of septicemia from doses of 0.5 c.c.

An attempt was made to find out how dangerous the animals with these chronic infections are to other animals.

In the first experiment 6 young healthy rabbits were put in the same cage with a rabbit having typical acute snuffles in the early stages, and with many typical bacilli in the discharge. To be sure of more intimate contact the noses of the normal rabbits were rubbed against the nose of the infected one. After 1 week none of the animals developed snuffles. Cultures from their nasal mucosa did not give positive results for the bacilli. Three of these animals were kept under observation for 2 weeks longer without developing the disease. The other 3 were now inoculated subcutaneously with 1 c.c. of a 24-hour culture of the bacilli. These all died in 3-5 days with typical symptoms and findings of septicemia.

Several similar experiments have been made by placing normal young animals in intimate contact with chronic nasal carriers described, but all these experiments have given negative results. In some cases the normal animals were permitted in the same cages with those infected for months (in 1 case, 7 months) and have remained healthy. Such contact animals do not apparently become carriers since the cultures made repeatedly from the nasal mucosa were always negative for the septicemia bacilli. From these experiments then it would seem that such chronically infected animals are not a source of danger to healthy animals by infecting them through the respiratory passages. However, the bacilli from these carriers, when injected subcutaneously, may give rise to typical abscesses, and if the dose is large enough septicemia will result. Through bites, therefore, and perhaps in other ways, these animals may be a source of danger. Every now and then it may be that a susceptible animal may acquire the disease through the respiratory tract, but no susceptible animals were detected in the experiments.

In addition to nasal carriers of the bacilli the animals which develop the subcutaneous abscesses also might be considered carriers of the disease. The abscesses are no doubt caused by infection through slight injuries received from the scratching or biting of an animal by one harboring a strain of the bacilli probably of reduced virulence in its mouth or on some other part. Experimentally, as noted, the injection of small doses of this organism reproduces the abscesses. It may not be necessary for the animal in question to be ill, since bacilli



of this type have been shown by Fiocca and others<sup>8</sup> to occur at times in the mouth and nose of apparently normal animals, which might serve then as healthy carriers. The abscesses are a chronic form of the infection where the bacilli exist often in pure form for a long time. They may rupture on the surface and discharge their contents, but often they do not. Entire removal of the encapsulated mass leads to complete recovery, and excision with discharge of contents will at times lead to recovery, but often a new abscess or several abscesses may reform in the vicinity. Bacteriologically, they are striking examples of a chronic subcutaneous focus of infection persisting at times almost indefinitely.

Whether these abscesses are dangerous to other animals and play a rôle in the direct transmission of the disease is doubtful. The bacilli from these abscesses when inoculated into the nose in quantities of several cubic centimeters do not develop septicemia. In 2 animals injected with large doses a mild acute rhinitis developed, with slight watery discharge from the nose for several days. I have also produced a small pneumonic lesion in the lung, but the characteristic acute pleuro-pericarditis and pneumonia I have not been able to cause by intranasal injection. With sufficiently large doses administered subcutaneously such lesions as well as general peritonitis and septicemia are readily produced. No doubt then these bacilli are very inferior in virulence and invasive properties to the organism met with in the respiratory passages and internal organs in the acute disease. However, by passing the organisms through only 2 or 3 animals by subcutaneous injection and reisolation from the blood or pericardium, the virulence is markedly raised and compares with that found in the natural disease in this respect.

When the virulent septicemia bacillus remains in an abscess for a time it loses its virulence, as is strikingly shown in the following experiment.

A rabbit was inoculated subcutaneously with 0.1 c.c. of an organism which had killed 2 rabbits by intranasal injection. An abscess the size of a large walnut appeared at the site, and remained with little change for 5 months. The organism was then reisolated from it, and 2 c.c. of a culture was inoculated intravenously into 2 rabbits without any results. At the end of 3 days a 2nd inoculation was made again without result. Eleven days after the 1st inoculation the animals were given intranasal inoculation with a freshly isolated bacillus from a sick animal, and both succumbed in 36 hours with septicemia. Culturally and morphologically, it should be said, the bacillus had retained all its original properties during its 5 months' sojourn in the abscess.

<sup>8</sup> Cited by Kolle and Wassermann, *Handb. d. path. Mikroorg.*, 1913, 6, p. 90.



In connection with these experiments there was opportunity to make observations on the resistance of the infected rabbits to subsequent infection, both natural and artificial, and also to study immunity reactions, especially on animals with the abscesses.

In the first place a vaccine was made with several strains of this organism, and a series of rabbits were injected for purposes of immunization. The vaccine was prepared by growing the bacillus on heavy blood agar for 24-36 hours, and suspending in 0.8% salt solution. After heating to 60 C. for 30 minutes, which kills the bacilli, the suspension was diluted so that 1 c.c. contained 100,000,000. Injection was made subcutaneously into a series of 7 rabbits using 100,000,000 per kilogram.

After the 7th injection the immunity of this series of rabbits was tested by inoculating them subcutaneously in the back with small doses of a 24-hour ascitic-broth culture recently isolated from the nasal discharges of an infected rabbit, which later died of rabbit septicemia. Six control animals were likewise inoculated. In 5 of the immunized series the dosage was 1 c.c. of the broth culture per kilogram of rabbit weight. In the other 2 a very much smaller dose (1 loop in 1 c.c. of salt solution) was given. On the 3rd day one of the former and on the 4th day another animal died, there being found at necropsy extensive infiltration into the muscles of the back with invasion of the lungs and blood stream. Of the 6 control rabbits 2 died, one on the 6th day after inoculation, another 3 days after inoculation. The findings in these animals were quite like those in the immune series.

All of the other rabbits, both the immunized and the controls, developed characteristic subcutaneous abscesses at or near the site of inoculation. No appreciable differences were noted between the 2 sets of animals. Those receiving the very small dose of 1 loop also developed abscesses which matured decidedly slower than those resulting from the larger doses.

All of the animals receiving the live bacilli in the course of the following 4 or 5 days lost 200-300 gm. Those in both series that lived, notwithstanding the abscesses, recovered their former weights in 3-4 weeks.

These animals then continued to live many weeks, nearly all of them gaining in weight. Inasmuch as rabbit influenza not uncommonly breaks out in the laboratory, the animals were carefully observed for any such outbreak. One of the control rabbits died of some undetermined cause some weeks later. Three months after the inoculation and while the abscesses still persisted, 2 of the 3 control rabbits developed typical snuffles, and from the nasal discharges the bacilli were found almost pure. Also at this same time in 4 of the immunized series, typical snuffles appeared and from the nasal discharge the organism was grown practically pure. Both series ran an identical clinical course which was not very severe, and no deaths occurred. In the cage containing the 4 immunized rabbits 1 of the animals became ugly and repeatedly bit the other 3 in various parts of their bodies, especially about the eyes. These wounds suppurated and the eyes became badly infected, and in 2 animals were completely closed from the swelling and exudate. From these lesions the bacilli were readily cultured in large numbers mixed with a few staphylococci.

Evidently protection against experimental inoculation or against natural infection was not obtained by the vaccination or through the presence of abscesses. It has already been noted that even the carrier state does not protect against the development of typical abscesses on

subcutaneous inoculation with small doses of the bacilli. Acute snuffles, however, did not develop in the 5 carrier rabbits while under observation, though they were repeatedly exposed.

In my previous paper I reported testing the serum for the presence of agglutinins in a number of rabbits having abscesses, of rabbits having snuffles and of guinea-pigs, after receiving several injections of dead bacilli. The results were all practically negative. I repeated the agglutination tests in the series of rabbits just referred to after they had been treated with 7 injections of dead bacilli. The results were practically negative, there being a trace of clumping only in dilutions of 1 : 20 in a few of the animals. Two strains, one isolated from the respiratory tract and the other from an abscess, were used.

Four of the animals of the immunized series that remained alive, after injection with living bacilli and abscess-production, developed snuffles which continued for several weeks. It was thought that these animals having received 7 doses of killed bacilli and having also the abscesses containing the live bacilli for several weeks and the respiratory infection for 4 weeks would show the antibodies, if any were formed against these bacilli. Agglutination tests made, however, even with autogenous strains, showed no appreciable clumping, either by microscopic or macroscopic methods.

With the serum of these same animals Dr. Moore, using the bacilli as antigens in varying concentration, was not able to detect any fixation of the complement.

The work of Chamberland and Jouan<sup>9</sup> on the interagglutination with the hemorrhagic-septicemia group was carried on with the serum of a highly immuned horse and showed with various strains from different animals including the rabbit, positive agglutination at dilutions varying from 1 : 10 to one to several thousand excepting with the homologous strain, where the agglutination appeared in dilutions of 1 : 60,000 to 1 : 80,000. These results, while clearly indicating the presence of antibodies in the immunized horse, are not directly comparable with the results given here.

In connection with some work carried out by Dr. Bernard Fantus on the problem of fever-production, several animals were treated with the heat-killed bacilli, and temperature and weight observations were made at suitable intervals.

The fever curve following such injections is quite definite and uniform, and occurs with a regularity that may make this organism

<sup>9</sup> Ann. de l'Inst. Pasteur, 1906, 20, p. 81.

a desirable agent for fever-production in the rabbit for experimental purposes. Fantus considers it the most reliable substance he has used and recommends it for testing the anti-pyretic action of drugs. Its fever-producing power is in all probability related to high degree of pathogenicity and virulence for the rabbit. There may be some difficulty encountered in producing this substance in bulk since the organism—at least certain strains—grows with considerable difficulty at times. By selecting suitable strains and using mediums rich in animal fluids this difficulty may be largely overcome.

The weight curve after the introduction into the rabbit of the dead organisms shows a typical and sharp decline followed by a more gradual rise. In the series of 8 rabbits injected with 100,000,000 per kilogram, following the 1st injection there was a drop in the weight curve of 200 : 300 gm. during the following week. Then usually appeared a more gradual return to the original weight, even though the animal received increasing doses at weekly intervals. Such animals apparently show an increasing tolerance to the repeated doses of this substance, which may be interpreted as a rough measure of an immunity reaction to the bacterial poisons. A similar phenomenon was noted in connection with fever-production.

#### SUMMARY

The well defined subcutaneous abscesses seen not infrequently in rabbits are caused by bacilli which are apparently identical with the rabbit septicemia bacillus. The virulence of the bacilli from the abscesses is markedly inferior to that of the organisms from the respiratory tract or internal organs.

On injecting a highly virulent bacillus in minute doses under the skin an abscess may form, remaining perhaps for many months. During this period the virulence of the bacilli is greatly decreased.

Rabbits after an attack of the disease and probably also without an attack become 'carriers' of the bacilli, both in the respiratory tract and subcutaneously in the abscesses. The virulence of the bacilli in these carriers is not high. However, organisms of low virulence of this group are known to rapidly increase their virulence under suitable conditions of transmission.

Rabbits treated with dead bacilli or having subcutaneous abscesses containing the bacilli are not immune to rabbit septicemia nor does their serum agglutinate the bacilli.

The heat killed bacilli on injection in suitable quantities into rabbits cause regularly a uniform fever curve.

# INHIBITORY ACTION OF LACTIC ACID ON CERTAIN BACTERIA AND FUNGI \*

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In his instructions for the cultivation of fungi and especially the isolation of pure strains, Duggar<sup>1</sup> recommends the use of lactic acid in the culture mediums. He claims that, in general, 0.5% of lactic acid is sufficient to prevent the growth of contaminating bacteria. As there is little or no available information concerning the inhibitory effects of lactic acid on any great variety of bacteria, although there is as to a few special forms, this work was undertaken with a view of determining the effect on common bacteria and certain fungi.

Apart from its bearing on methods of cultivation, there is possibly a more important relation between bacteria and lactic acid. Under pathologic conditions lactic acid is the most widely distributed and abundant acid in the body. It is found in the tissues, in the secretions of the stomach, in abscesses, and in various fluids of the body. It is asserted by Fischer that an increased production of acids in the tissues is responsible, to a large degree, for the occurrence of edema. Therefore, since lactic acid is so commonly found in the body, it would seem that the action of this acid on bacteria might be a truer test of the tolerance of bacteria toward acid, that is toward the effect of the hydrogen-ion, than would be that of an inorganic acid like hydrochloric, as we are more nearly approximating common pathologic conditions found in the body.

The method employed in these preliminary experiments was as follows:

Plain standard agar was prepared, Armour's peptone being used because of the inability to procure Witte's. This was carefully titrated so that after 1 hour of sterilization in the autoclave at 15 pounds' pressure it had a neutral reaction to phenolphthalein. Ten c.c. were placed in each tube. The lactic acid was used undiluted, 0.1 c.c. equaling 1% of lactic acid, and corresponding amounts for the varying percentages of acid. For the fungi and higher bacteria, a neutral 1% glucose agar was used.

\* Received for publication June 2, 1917.

<sup>1</sup> Fungous Diseases of Plants, 1909.



The tubes of agar were melted and cooled to about 50 C., and the varying amounts of lactic acid added and mixed by careful rotating. When cooled to about 40 C. they were inoculated with 1 loopful of the organism from a 24-hour culture. This was mixed by rotating so that all the bacteria would come in equal contact with the acid and then slanted. Ordinarily 24 hours was sufficient for growth at 37 C., although in some cases with higher acid-content growth did not appear for 48 hours. Maximum growth with bacteria was generally attained in 48 hours. After 48 hours, the cultures were kept at room temperature for 7 days, but in no case was further growth appreciable. The higher bacteria and fungi generally required a longer time for development.

It was found that the addition of the lactic acid to the melted agar and the inoculation with the organisms while the medium was still liquid gave the best results, and also insured the constancy of the acid. The lactic acid-content of the medium was titrated with N 10 KOH, using the method of Harada,<sup>2</sup> and it was found that the amount of lactic acid as shown by neutralization was the same as had been added. However, if the acid were added to the unmelted agar and then boiled the lactic acid might be diminished.

In the present experiment, only 1 strain of each organism, except in the case of the streptococci to be mentioned later, was employed. Twenty-five different bacteria including most of the ordinary organisms were used, also 6 of the higher organisms.

TABLE 1  
PERCENTAGE OF LACTIC ACID INHIBITING BACTERIAL AND FUNGUS GROWTH

	%		%
<i>B. diphtheriae</i> .....	0.1	<i>Streptococcus viridans</i> .....	0.4
<i>Spirillum Finkleri</i> .....	0.2	<i>B. Tuberculosis</i> .....	0.5*
<i>Spirillum Metchnikovii</i> .....	0.2	<i>B. lactis aerogenes</i> .....	0.5
<i>Sarcina lutea</i> .....	0.2	<i>B. enteritidis</i> .....	0.6
<i>B. typhosus</i> .....	0.2	<i>B. fecalis alcaligenes</i> .....	0.6
<i>B. dysenteriae</i> .....	0.3	<i>B. prodigiosus</i> .....	0.9
<i>B. cholerae-suis</i> .....	0.3	<i>B. pyocyaneus</i> .....	0.9
<i>B. paratyphosus A</i> .....	0.3	<i>B. violaceus</i> .....	0.9
<i>B. paratyphosus B</i> .....	0.3	<i>B. subtilis</i> .....	1.2
<i>Proteus vulgaris</i> .....	0.3	<i>B. mucosus</i> .....	1.5
<i>B. cloacae</i> .....	0.3	<i>Actinomyces bovis</i> .....	2.3
<i>Staphylococcus albus</i> .....	0.3	<i>Sporothrix schenckii</i> .....	2.8
<i>Staphylococcus citreus</i> .....	0.3	<i>Blastomyces</i> .....	2.9
<i>Staphylococcus aureus</i> .....	0.4	<i>Mucor mucedo</i> .....	7.6
<i>B. coli</i> .....	0.4	<i>Aspergillus flavus</i> .....	8.6
<i>B. rhinoscleromatis</i> .....	0.4		

\* Grown on glucose agar, 3%.

It was found that the majority of the organisms was inhibited with less than 1% of lactic acid. It is interesting to note that of the 2 organisms resisting 1% of acid, one was a spore-former, *B. subtilis*, and the other a capsulated organism, *B. mucosus*. The higher bacteria, including *Actinomyces*, *Sporothrix*, and *Blastomyces* were more than twice as resistant as the other bacteria, while the 2 fungi, *Mucor mucedo* and *Aspergillus flavus*, were decidedly tolerant, growing in a medium containing several per cent. of acid.

<sup>2</sup> Am. Jour. Med. Sci., 1916, 152, p. 243.



In Table 1 the figures represent the highest acid-content at which growth appeared, growth being totally inhibited with a greater acid-content. Growth, irrespective of its amount, is alone considered.

It was found on plotting a curve of the percentages of acid-toleration that the first portion of the curve which included the larger number of organisms tested was nearly a straight line, deviating only slightly from the perpendicular. There was a decidedly sharper curve in that portion which represented the acid resisted by the higher forms. However, it is probably true that if a sufficient number of the higher bacteria and fungi were tested the curve would approximate a straight line.

A series of tests were made using 12 different strains of streptococci, 5 being isolated from milk, the remainder from pathologic conditions. It was found that a wide range of tolerance to the acid existed in both groups, one organism being barely resistant, and some being among the most resistant of all the bacteria tested. However, when the average of these values was obtained, it was found not to be essentially different from what might have been expected of this type of organism as judged by other related bacteria. Harada<sup>2</sup> likewise found a varying tolerance of different strains of streptococci to lactic acid.

It is to be supposed that had different strains of other organisms obtained from a variety of sources been tested, a similar range of tolerance would have been found, but probably the average of these values would not have essentially affected the general form of the curve.

It was also found that related types of bacteria or those belonging to the same group, as for example, the staphylococci or the group containing *B. typhosus*, *B. dysenteriae*, and *B. cholerae-suis*, exhibited the same or only slightly varying tolerance. It is interesting to consider whether or not tolerance toward lactic acid, especially in pathogenic organisms, may not be as individual a characteristic as is the behavior of bacteria toward the various sugars.

The fact was brought out that the group of chromogenic bacilli, *B. prodigiosus*, *B. pyocyaneus*, and *B. violaceus*, which were all quite resistant to the acid, exhibited a progressively increasing amount of chromogenesis in direct proportion to the amount of acid present up to the limits of toleration. Jordan<sup>3</sup> in his work with *B. pyocyaneus* found that the presence of lactate in the medium increased pigment-

<sup>2</sup> Jour. Exper. Med., 1899, 4, p. 627.

production. It is undoubtedly due to the negative radical of the lactic acid rather than to the hydrogen-ion that the increasing pigment is produced. It has not apparently been noted heretofore that the presence of lactic acid or any of its salts has the same effect on *B. prodigiosus*, *B. violaceus*, or other chromogens.

#### SUMMARY

Lactic acid has a varying degree of inhibitory action on the various bacteria and fungi, the action being apparently less on the more complex forms than on the simpler bacteria.

In the majority of cases, 24 of 31 organisms, growth was inhibited with less than 1% of lactic acid.

Twelve strains of streptococci from a variety of sources, including milk and different pathologic conditions, were tested, and gave a wide range of tolerance; but the average of these values was 0.6%.

With chromogenic bacteria, pigment-production increased progressively up to the limits of tolerance.



# SURFACE STERILIZATION OF TISSUES FOR BACTERIAL STUDIES

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In a series of experiments for the purpose of studying the bacterial content of the prostate, certain difficulties were encountered with the methods commonly described for making cultures from tissues. It was found that by using the method recommended by Rosenow<sup>1</sup> certain discrepancies occurred in the results which suggested that they may have been due to contamination introduced at some point between the time the tissue was exposed by the surgical scalpel and the final closure of the culture tube. In order to decide this question decisively it was determined to put a close check on each step in this process.

Within the last few years a great deal of interest has been taken in the bacteriology of glandular tissues and its connection with the etiology of disease. Negri and Mieremet<sup>2</sup> and Bunting and Yates<sup>3</sup> almost simultaneously isolated a pleomorphic diphtheroid bacillus from the glands in Hodgkin's disease. Later Billings and Rosenow<sup>4</sup> were also successful in cultivating a diphtheroid bacillus in this condition. Rosenow,<sup>5</sup> Rhea and Falconer,<sup>6</sup> Bloomfield,<sup>7</sup> Torry,<sup>8</sup> Langford,<sup>9</sup> Cunningham,<sup>10</sup> Wade and Harris,<sup>11</sup> Fox<sup>12</sup> and others have also reported the recovery from Hodgkin's disease of several different types of diphtheroid organisms, pigmented and nonpigmented, and associated with these they found various organisms as contaminators; e. g., staphylococcus albus and aureus, streptococcus, *B. welchii* and others. Bunting and Yates<sup>3</sup> say that "although the utmost efforts were made to prevent carrying in organisms from the skin when removing nodes for cultural investigation, in almost every case studied, one or more tubes have shown the presence of a white staphylococcus." Torry<sup>8</sup> in a study of normal and abnormal lymph nodes found a diphtheroid bacillus of one type or another from 22 of 40 cases in such

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<sup>1</sup> Centralbl. f. Bacteriol., I, O., 1914, 74, p. 366; Jour. Am. Med. Assn., 1914, 63, p. 903.

<sup>2</sup> Centralbl. f. Bacteriol., I, O., 1913, 67, 292.

<sup>3</sup> Arch. Int. Med., 1913, 12, p. 236; Jour. Am. Med. Assn., 1913, 61, p. 1803; *ibid.*, 1914, 62, p. 516; *ibid.*, 1914, 62, p. 177.

<sup>4</sup> Jour. Am. Med. Assn., 1913, 61, p. 2122.

<sup>5</sup> Jour. Am. Med. Assn., 1914, 63, p. 903.

<sup>6</sup> Arch. Int. Med., 1915, 15, p. 438.

<sup>7</sup> Arch. Int. Med., 1915, 16, p. 197.

<sup>8</sup> Jour. Med. Res., 1916, 29, p. 65.

<sup>9</sup> Am. Jour. Trop. Dis. and Prev. Med., 1914, 2, p. 191.

<sup>10</sup> The Am. Jour. Med. Sc., 1917, 153, p. 406.

<sup>11</sup> Jour. Exp. Med., 1915, 21, p. 493.

<sup>12</sup> Arch. Int. Med., 1916, 16, p. 465.

divers pathologic conditions as Hodgkin's disease, chronic hyperplastic lymphangitis of obscure nature, lymphosarcoma, sarcoma, melanoma, endothelioma, tuberculous adenitis and chronic lymphatic leukemia. The organisms were studied and grouped. Animal inoculations with these organisms were made in monkeys and the results were negative. Again, he recovered as many as 5 distinct types of diphtheroids in a single case of Hodgkin's disease. Tests made for agglutinins were negative. In conclusion, he says "cultural findings in various types of abnormal gland have indicated that this bacillus does not stand in specific relationship to any definite pathologic condition." This is substantiated by agglutination and complement fixation experiments with the blood of the patients. As he has found a bacillus of such uniform type and occurring so frequently in abnormal states of lymph gland he suggests the name of *B. lymphophilus*. Rosenow,<sup>1, 2</sup> of 54 cases of arthritis deformans, isolated a nonhemolyzing streptococcus 32 times, staphylococcus 5 times, *B. welchii* in 14 cases, an organism resembling *B. mucosus* 3 times, *M. catarrhalis* and the gonococcus once each, and diphtheroid bacillus in 5 cases; in 7 cases the cultures remained sterile. In 7 cases of erythema nodosum excised nodes from the cervical lymph glands gave a polymorphous, sometimes clubbed diplobacillus. Out of 32 cases of goiter in man, he isolated an anaerobic gram-positive diplobacillus-like organism from the thyroid gland. And in 8 out of 12 dogs having goiter *B. welchii* was found in all but 6 of the thyroid glands. A hemolytic staphylococcus was found in most goiters, in man and dog. In another report,<sup>13</sup> he obtained positive results from glands only 5 mm. in diameter. The number of colonies ranged from one to two thousand. In 38 cases he isolated organisms from all but 3 cases. Streptococcus was obtained in 14 cases. *B. welchii* was obtained in 9 cases, staphylococcus in 3 cases and gonococcus in one. Cunningham,<sup>10</sup> in his gland cultures obtained in some cases pure cultures of staphylococcus in some of the tubes. Finch<sup>14</sup> isolated a sporothrix from axillary glands.

A number of these investigators<sup>8, 10, 12</sup> have been unable to identify by serological reaction the organisms recovered by them with that described by Bunting and Yates. In addition Torry, Fox, Cunningham, and Cellar (as reported by Libman<sup>15</sup>), have found diphtheroid bacteria in tuberculous and other glands not the seat of Hodgkin's disease Harris and Wade<sup>11</sup> have shown that diphtheroids are widely distributed in nature, as they recovered them from the air, body surfaces, and at times from deep tissues so that they concluded that they were there through contamination or else they are indigenous in these locations. Ford<sup>16</sup> and Nicolle<sup>17</sup> have stated that they are present in normal tissues, and in pathologic conditions to which they bear no etiologic relation, as in lesions of leprosy, blastomycosis, tertiary syphilis, and tumors of various types, as did Torry, Cunningham and Cellar. Harris and Wade<sup>11</sup> also cite the fact that organisms have been isolated from human tissue, removed with presumably sterile precautions. Wolbach and Saike<sup>18</sup> isolated an anaerobic spore-bearing bacillus from apparently normal animals in 21 of 23 cases.

In spite of the great quantity of work done the divergent results still leave open the question as to the relations the organisms isolated bear to the diseases in question.

<sup>13</sup> Jour. Am. Med. Assn., 1914, 62, p. 1146.

<sup>14</sup> Proceedings New York Path. Soc., 1914, 14, p. 141.

<sup>15</sup> Jour. Am. Med. Assn., 1914, 63, p. 907.

<sup>16</sup> Trans. Assoc. of Am. Physicians, 1900, 15, p. 389.

<sup>17</sup> Canad. Jour. Med. and Surg., 1899, 6, p. 405.

<sup>18</sup> Jour. Med. Res., 1909, 21, p. 267.



## SUMMARY OF PREVIOUS METHODS

Steele<sup>19</sup> grinds up inguinal glands in a sterile mortar but no further particulars are given. Langford<sup>9</sup> uses the bunsen flame. Fox<sup>12</sup> dips the tissue in hot oil (length of time not given), transfers it to ether, to salt solution and then to a large sterile bottle where it is cut up with scissors. Torry<sup>8</sup> sterilizes the surface by "dipping momentarily in boiling water and then transferring quickly to cool sterile saline solution. The gland is then placed in a sterile dish and macerated under a glass cover with fine scissors, not over five minutes elapsing, as a rule, during this procedure." Finch<sup>14</sup> "keeps the glands sterile," but does not describe how it is done. Rhea and Falconer<sup>3</sup> do not describe any method of sterilization. Bloomfield<sup>7</sup> washes the

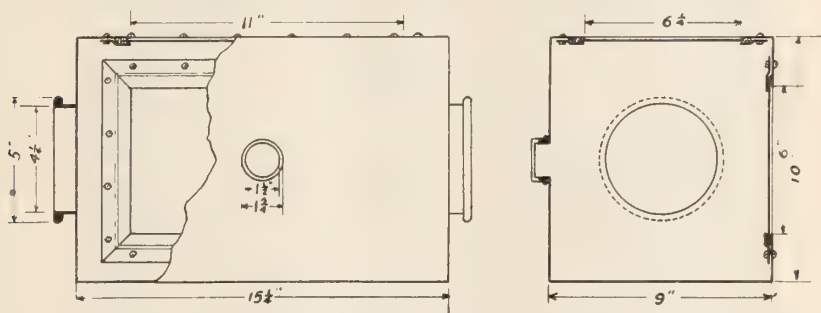


Fig. 1. Scale drawing of air chamber. Note construction of small hole for introduction of tissue. Edge of cap need not touch edge of hole, which is one-fourth inch smaller all around.

tissue several times in sterile salt solution, then dips it in boiling salt solution, the lengths of time varying with the size of the tissue. Rosenow<sup>1</sup> says, "Zur Sterilisierung der Aussenflächen werden dieselben durch eine Bunsenflamme gezogen oder für kurze oder längere Zeit in kochendes Wasser getaucht; der Zeitraum für die letztere Prozedur is von der Grösse der Gewebestückes abhängig, worauf die so sterilisierten Gewebe sofort in kalte, sterile Kochsalzlösung gelegt werden." In a later publication,<sup>5, 20</sup> he further states that he receives the tissue in gauze and carries it to the laboratory, emulsifies it, after sterilization, in broth or salt solution in a mortar in a specially devised sterile chamber. Cunningham<sup>10</sup> brought the glands to the laboratory in a sterile towel, 'mashed or squeezed' them and then placed them on

<sup>19</sup> Boston Med. and Surg. Jour., 1914, 170, p. 123.

<sup>20</sup> Jour. Inf. Dis., 1915, 16, p. 367.

various media. The instruments used were from the autopsy room, hastily boiled up. With this method various organisms which the author thinks were due to contamination, were obtained. Later improvements in the technic, the details of which are not specified, reduced the number of positive cultures. The only reference to the length of time used in surface sterilization made by any authors consulted is that it varied with the size of the tissues.

#### DESCRIPTION OF THE APPARATUS

Owing to the fact that the air chamber recommended by Rosenow as found on the market is not air-tight; that it is very unstable; that



Fig. 2. Larger half section drawing of small hole for introduction of tissue.

only one hand can be introduced and that the large cotton plug used is difficult to manage, it was decided to design a new chamber to eliminate these difficulties. The chamber, as finally constructed, is rectangular and made of heavy tin with large panes of plate glass at both the top and front, a hand-hole at each end, to which canvas gloves are attached, so that both hands can work on the inside of the box, and an opening on the metal side for introducing the necessary materials and tissue. As can be seen by referring to the accompanying diagram, the flange of this opening is one-fourth inch smaller than the cap, so that the edge of the cap never comes in contact with the flange. This eliminates the cotton plug.

## TESTS OF APPARATUS

In order to make sure that this apparatus accomplished the objects for which it was designed, tests were made, cultures of pigment-producing bacteria having been previously placed on the tissues and hands, according to the following scheme:

Tissue that has been autoclaved is cut into pieces of desired size, approximately  $1.5 \times 2 \times 1.5$  cm., in a sterile jar, dipped in a 24 hour broth culture of *Staphylococcus aureus*, and the methods of surface sterilization to be subsequently described carried out. One piece of

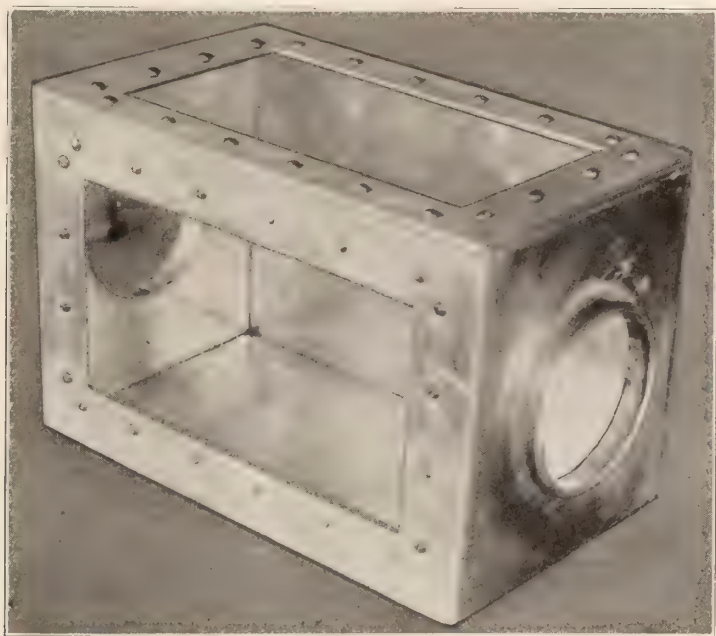


Fig. 3. Air chamber, showing glass windows and hand-holes.

the tissue is boiled 20 seconds in salt solution, another piece is flamed for 20 seconds, and a third piece is placed in oil at 180 C. for 5 seconds. The tissue is then dropped through the small opening into a mortar in the airchamber, to which a large test tube of dextrose ascitic broth<sup>1</sup> has previously been added (flaming the opening each time it is opened and closed). The chamber contains mortars, pestles, quartz sand, a block of wood holding four large test tubes, scissors and forceps, the whole having been sterilized for one hour at 160 C. each time before

use. The hands are washed for 10 minutes in soap and water, rinsed several times in bichlorid 1:1000, then in distilled water and lastly in sterile salt solution. The hands now being nongermicidal and relatively sterile, are dipped in a broth emulsion of a 24 hour culture of *B. violaceus* and introduced into the sterile canvas gloves. The tissue is then cut up into very fine pieces, ground for 15 minutes, quartz sand is added and the grinding continued for 15 minutes longer. This emulsion is poured into one of the large test tubes which is then removed and after washing the hands plates of the emulsion are made, using 1 c.c. for each plate.

With this method all the plates remained sterile, neither *Staph. aureus*, *B. violaceus* nor any other organisms growing upon them.

Having demonstrated that the air chamber served to prevent contamination of the tissue, it was now necessary to check the method used for the surface sterilization of the pieces of tissue, which are usually obtained from the operating room. To this end, previously sterilized tissue inoculated on the surface or the interior with known organisms, was used.

#### OUTLINE OF METHOD

1. Mortars or evaporating dishes and pestles, scissors and tissue forceps are placed in a wire basket and sterilized in the hot air oven for one hour at 190 C., the mortars or dishes being upside down.

2. The tissue to be used, preferably kidney or liver, is autoclaved, placed in a sterile glass jar containing tissue forceps and scissors, and cut into pieces about 1.5x2x1.5 cm. in size.

3. A piece of tissue is dipped in an emulsion of a 24 hour broth culture of the organism for an instant; that is, just enough to cover the surface.<sup>21</sup>

4. The tissue is removed and placed in hot oil (liquid paraffin) boiling salt solution or a Bunsen flame the desired length of time.

5. It is dropped at once into the sterile mortar to which has been previously added 10 c.c. of sterile broth, and ground with the sterile

<sup>21</sup> It is understood that this is an exaggeration of the infection usually present on tissues to be cultured, but that such infection is not negligible is shown by a recent culture of 10 c.c. of broth into which a piece of prostate, dropped by the operator into a sterile glass jar, had been placed for a moment. A plate made with 1 c.c. contained 250 colonies. In another case, 1 c.c. of broth in which the prostate had been immersed produced 250 colonies, whereas after immediate surface sterilization for eight seconds in liquid paraffin at 180° C., emulsifying the tissue, in which a pus cavity was found, and plating 1 c.c. of this emulsion, the colonies were innumerable.

pestle, thus breaking up the tissue. The air chamber was used in the first few series but was deemed unnecessary for these thermal experiments.

6. One c.c. of this tissue emulsion is removed and plated. Repeat this procedure, changing the time of heating and media as desired.

7. As a check on the tissue used, a piece which has not been inoculated is emulsified and 1 c.c. of this emulsion is plated.

8. As a check on the organism and to have a culture where no sterilizing methods had been used with which to compare the growth

TABLE 1  
TESTS WITH BOILING 0.85 PER CENT. NaCl SOLUTION

Tissue Inoculation* on	Length of Time Tissue Was Heated in Boiling Saline Solution							
	5 sec.	10 sec.	15 sec.	20 sec.	25 sec.	30 sec.	40 sec.	45 sec.
Exterior	+	+		0		0		
Exterior	+	+		0		0		
Exterior	+	+		0		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior	+	+		+		0		
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0		0
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior		+		+		0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		+	0	0	
Exterior and interior			+		0	0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	0	
Exterior and interior			+	+		0	?	

\* *Staphylococcus aureus* was used to inoculate the tissue.

+ = abundant growth; 0 = no colonies; ? = a few colonies.

from the heated tissue, a piece of tissue is dipped in a bacterial emulsion and then run through like the pieces which have been heated.

9. For deep tissue inoculation a hypodermic syringe is used, the bacterial emulsion being forced into the interior of the tissue and the whole then dipped in the same emulsion. The procedure from this point is the same as above. In this way the amount of heat necessary to destroy the surface bacteria without interfering with the organisms that are on the interior can be determined.



## DISCUSSION

1. Boiling salt solution. The length of time required to destroy organisms on the surface of pieces of tissue dipped in suspensions of *Staphylococcus aureus* lies between 20 and 25 seconds, as growth was obtained after heating for 20 seconds in 12 of the series. In 3 series no growth occurred after heating 20 seconds. It made very little difference whether the tissue had been inoculated on the inside or the

TABLE 2  
TESTS WITH BUNSEN FLAME

	Tissue Inoculated* on	Length of Time Tissue Was Kept in Bunsen Flame						
		1 sec.	3 sec.	5 sec.	10 sec.	20 sec.	25 sec.	30 sec. 40 sec.
I	Exterior	+	+	+	+	?		
II	Exterior	+	+	+	+			
III	Exterior	+	+	+	+			
IV	Exterior	+	+	+	+			
V	Exterior	+	+	+	+			
VI	Exterior	+	+	+	+			
VII	Exterior	+	+	+	+			
VIII	Exterior	+	+	+	+			
IX	Exterior	+	+	+	+			
X	Exterior	+	+	+	+			
XI	Exterior				+	+	0	0
XII	Exterior				+	+	0	0
XIII	Exterior				+	+	0	0
XIV	Exterior				+	+	0	0
XV	Exterior				+	?	0	0
XVI	Exterior				+	+	0	0
XVII	Exterior				+	0	0	0
XVIII	Exterior				+	+	0	0
XIX	Exterior				+	+	0	0
XX	Exterior				+	+	0	0
XXI	Exterior				+	?	0	0
XXII	Exterior				+	+	0	0
XXIII	Exterior				+	+	0	0
XXIV	Exterior				+	?	0	0
XXV	Exterior				+	+	0	0
I	Exterior and interior				+	+	0	0
II	Exterior and interior				+	+	0	0
III	Exterior and interior				+	+	0	0
IV	Exterior and interior				+	+	0	0
V	Exterior and interior				+	+	0	0
VI	Exterior and interior				+	+	0	0
VII	Exterior and interior				+	+	0	0
VIII	Exterior and interior				+	+	0	0
IX	Exterior and interior				+	+	0	0
X	Exterior and interior				+	+	0	0

\* *Staphylococcus aureus* was used to inoculate the tissue.

+ = abundant growth; 0 = no colonies; ? = a few colonies.

surface; in other words, if sufficient heat was applied to destroy surface bacteria, the organisms on the interior were also killed. The boiling salt solution method, then, leaves no margin of safety.

2. The results in Table 2 are even more surprising. There is growth after heating the tissue 20 seconds in a Bunsen flame above the cone, turning the tissue continually so that the flame will reach every

part. This is sufficient to char the tissue. In 10 series above 10 seconds was required, but not realizing that it would require such a length of time to destroy the organisms in the flame, no tests were made at longer than 10 seconds. In 15 other series the thermal death point was found to be above 20 seconds. This is sufficient to destroy the organisms on the interior as well. The only way that this can

TABLE 3  
TESTS WITH LIQUID PARAFFIN

Series	Tissue Inoculated* on	Temp. of Oil	Length of Time Tissue Was Heated in Oil				
			2 sec.	5 sec.	10 sec.	15 sec.	20 sec.
I	Exterior	150 C.	+	+	+		
II	Exterior	150 C.	+	+	+		
III	Exterior	150 C.	+	+	+		
IV	Exterior	150 C.	+	+	+		
V	Exterior	180 C.		+	0	0	
VI	Exterior	180 C.		+	0	0	
VII	Exterior	180 C.		0	0	0	
VIII	Exterior	180 C.			0	0	
IX	Exterior	180 C.	+	+	0		
X	Exterior	180 C.	+	+	0		
XI	Exterior	180 C.	+	0	0		
XII	Exterior	180 C.	+	0	0		
XIII	Exterior	180 C.	+	0	0		
XIV	Exterior	180 C.	+	+	0		
XV	Exterior	190 C.	+	+	0		
XVI	Exterior	190 C.	+	0	0		
XVII	Exterior	190 C.	+	+	0		
XVIII	Exterior	190 C.	+	+	0		
XIX	Exterior	190 C.	+	0	0		
XX	Exterior	200 C.	0	0	0		
XXI	Exterior	200 C.	+	?	0		
XXII	Exterior	200 C.	+	?	0		
XXIII	Exterior	200 C.	+	0	0		
XXIV	Exterior	200 C.	+	0	0		
XXV	Exterior	200 C.	+	?	0		
I	Exterior and interior	180 C.		+	+		0
II	Exterior and interior	180 C.		+	+		0
III	Exterior and interior	180 C.		+	+		0
IV	Exterior and interior	180 C.		+	+		0
V	Exterior and interior	180 C.		+	+		?
VI	Exterior and interior	180 C.		+	+		0
VII	Exterior and interior	190 C.		+	+		0
VIII	Exterior and interior	190 C.		+	+		0
IX	Exterior and interior	190 C.		+	+		0
X	Exterior and interior	190 C.		+	+		0
XI	Exterior and interior	200 C.		+	0		0
XII	Exterior and interior	200 C.		+	0		0
XIII	Exterior and interior	200 C.		+	0		0
XIV	Exterior and interior	200 C.		+	0		0

\* *Staphylococcus aureus* was used to inoculate the tissue.  
+ = abundant growth; 0 = no colonies; ? = a few colonies.

be explained is by assuming that the charred tissue protects the bacteria, acting as a nonconductor of heat, or that the flame does not reach every part of the tissue.

When experiments with prostatic gland tissue were begun oil was considered a desirable medium. Its previous use was not known at that time, but it has since been learned that Fox<sup>12</sup> used oil in sterilizing tissues, as already mentioned.

3. In Table 3, where liquid paraffin was used at various temperatures and times, it was found that at 150 C. growth was still obtained at the end of 10 seconds in 4 series, so that the thermal death point for this temperature in these series was not definitely determined, while at 180 C. for 4 series the thermal death point was above 2 seconds but below 5 seconds, and for 6 series it was above 5 seconds and below 10 seconds. At 190 C. the thermal death point was practically the same as that at 180 C., while at 200 C. the thermal death point was below 2 seconds for 1 series, above 2 seconds for 2 series and above 5 seconds for 3 series. For the series where the organisms were

TABLE 4  
THE GREATEST LENGTH OF TIME AT WHICH ANY GROWTH WAS OBTAINED IN  
EACH SERIES IS INDICATED  
Boiling .085 percent. NaCl Solution

T. D. P. for Surface Organisms. Under		T. D. P. for Interior Organisms. Above		
20 seconds	30 seconds	15 sec.	20 sec.	25 sec.
3 series	12 series	1 series	6 series	3 series

Bunsen Flame

Undetermined*	20 seconds	25 seconds	30 seconds	20 seconds
10 series	1 series	9 series	5 series	10 series

Liquid Paraffin

Temp. of Paraffin	Below 2 seconds	2 seconds	5 seconds	10 seconds	5 seconds	10 seconds
150 C.	0	0	0	4 series		
180 C.	0	4 series	6 series	0	0	6 series
190 C.	0	2 series	3 series	0	0	4 series
200 C.	1 series	2 series	3 series	0	4 series	0

\* In these ten series, the T. D. P. was somewhere above 10 seconds.

inoculated on the interior as well as on the exterior the thermal death point for 180 C. was above 10 seconds in 6 series, for 190 C. it was the same for 4 series and was found to be above 5 seconds for 4 series at 200 C.

The use of oil at 190 C. and 200 C. is accompanied by some inconvenience due to the ebullition of the oil at the time the tissue is introduced. This is not the case at 180 C. This series of experiments demonstrates that if a piece of tissue of the size used is dipped in oil at 180 C. for between 5 and 10 seconds, probably best at 6 seconds, bacteria on the surface will be destroyed without injuring the organisms

on the interior. It may be that if the tissue is obtained under rigid precautions and the outside washed several times in salt solution (as suggested by Bloomfield<sup>7</sup>) a shorter period would be allowable if one were dealing with a very thermo-sensitive organism on the interior of the tissue.

To procure pieces of tissue from the living body without contamination of the surface of these pieces is beyond doubt extraordinarily difficult. Great precautions must be taken in removing the tissue for the preparation of spirochaete culture media, where no other object is in view. In surgical operations, where searing of the skin, etc., cannot be practiced, the liability to contamination is increased. These considerations have guided previous bacteriologic workers in their efforts to sterilize the surface of the tissue, by methods which are here shown to be of doubtful value. In using the oil method, which is the best we have experimented with, one undoubtedly runs a risk of destroying delicate organisms within the tissue, although practically certain of freedom from surface contamination. But if any less effective method is used it is impossible to be sure that surface contamination has not influenced the results.

#### SUMMARY

With the air chamber and technic described in this paper danger of contamination from the outside during the making of cultures from tissues is reduced to the minimum.

It is apparent that the boiling water method as formerly used is inadequate for surface sterilization, and that the time required to sterilize the surface with certainty approaches that sufficient to sterilize the gland completely.

The same objections apply to the Bunsen flame. The flame either does not reach every part of the tissue, or the charred tissue acts as a nonconductor of heat.

The hot oil method of sterilization answers the purpose better than the preceding two methods, as at a temperature of 180 C., surface bacteria will be destroyed in 5 seconds without apparently interfering with the organisms in the interior of the tissue, which are not killed unless it is heated for more than 10 seconds.

In view of the stress laid on the bacteriology of glandular tissues in relation to the etiology of disease, it becomes apparent at once that surface sterilization is of the utmost importance. Having obtained a satisfactory method of surface sterilization, more confidence can be placed in the results obtained in future experiments.

# COCCOBACILLUS (FOETIDUS) OZAENAE OF PEREZ \*

PLATES 1 AND 2

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The coccobacillus of Perez is of practical interest because of its supposed rôle in the ozena stage of atrophic rhinitis. It has been isolated from ozena by Horowitz, Perez, Hofer, and in this country by Horn and Ward.<sup>1</sup> So far the reports have not dealt extensively with the characteristic of the organism, and in this article I wish to give briefly the principal results of a study of several strains both from European sources and obtained here from patients.

Nasal exudates are inoculated in broth tubes, which are incubated for 15-20 hours, when subcultures are made on other mediums. I have found that this is a good method because the Perez bacillus practically outgrows the other organisms introduced into the broth. The following statements are based on the detailed study of 12 strains selected from 50 isolated altogether.

The bacillus is small, varies in diameter, the ratio of breadth to length being 1:1-1:4. Some of the strains assume distinctly bacillary forms on all mediums while others are often coccoid. Coccoid forms occur especially in liquid medium and in mass growths; on potato and solid mediums atypical forms are frequent. The bacillus forms no chains, no spores, is gram-negative, but otherwise stains readily. The European strains are nonmotile, and this is the case also with strains isolated here, although some appear to have become motile on cultivation, while others seemingly have been sluggishly motile from the first.

The organism is easily cultivated. Agar colonies are moist, round, translucent, bluish pale, about 2 mm. in size, the center after a few days assuming a yellow-brown tinge. On agar slants luxuriant growth develops, moist, translucent, with wavy borders, faint yellow-brown streaks appearing in older cultures, without odor. Agar stabs give a good growth, without pigmentation as a rule, the growth at the end of the puncture being slight. After 5 days at 37 C., gelatin stabs develop a uniform turbidity without liquefaction. On blood serum the growth is moist, glistening, faintly yellowish, and without odor. On potato there develops a moderate, moist, gray or faintly yellow growth, without odor. Broth becomes turbid in a few hours; in old cultures a delicate blue film forms on the surface and an unpleasant nauseating odor develops, different from that produced by *B. proteus*, *B. coli*, and the cocci. A small amount of acid is produced in litmus milk after 7 days, without clotting. Dextrin, levulose, lactose, saccharose, maltose, glycerol, inulin, and mannite are

\* Received for publication May 7, 1917.

<sup>1</sup> Jour. Infect. Dis., 1916, 19, p. 153. For other references see this article.



not fermented; glucose alone is reduced, but fresh cultures may not act until 3-5 days; after 10 days the amount of gas produced may run 15-75%; the ratio of  $\text{CO}_2:\text{H}::2:3$ . In broth to which litmus, methylene blue, or neutral red have been added, there is complete reduction in 2-4 days.

In deep gelatin colonies pigment develops, so that after 24-72 hours the center of the colonies, by transmitted light, appears as a mahogany brown, lobulated mass within a lighter brown zone, and after 4-5 weeks at room temperature the colonies may appear as black dots on a white background. Occasionally old broth cultures develop a color so that they look like turbid claret.

The action of the bacillus on protein material is characteristic. In Dunham medium peptones are broken up and indol, skatol, ammonia, hydrogen sulphid, methylmercaptan, and other by-products are formed. The same sort of decomposition occurs also in broth, the process being only partly interfered with by carbohydrates.

Undoubtedly the odor in ozena and the odor of broth cultures of Perez' bacillus is due to volatile sulphur compounds.

In rabbits the intravenous injection of culture filtrates may cause death in 48 hours, and the injection of killed and washed bacilli may give rise to ulceration. No evidence of the formation of a true bacterial toxin has been obtained. The intravenous injection of living cultures in rabbits produces, as shown by Perez and others, increased secretion from the nose, a thin watery discharge developing, which later becomes mucopurulent. It is possible to establish in this way a chronic condition, with crusts about the nostrils. The bacillus is easily recovered from the nose, and may be recovered from the blood, spleen, and other organs. When large doses are given, death may occur within a short time; after smaller doses, death may occur after several weeks, preceded by emaciation. Some rabbits are immune.

TABLE 1

INTRAVENOUS INJECTIONS OF SALT SUSPENSIONS OF PEREZ' BACILLUS

Strains	Dose, C.c.	Nasal and General Reactions	Result
500	2.5	Marked nasal secretion	Died in 24 hours
500	2.0	Nasal crusts, emaciation	Died in 42 days
500	2.0	Nasal crusts, emaciation	Died in 32 days
500	2.0	Nasal crusts, emaciation	Died in 17 days
12.2	3.0	Moderate secretions	Died in 15 hours
12.2	2.0	Moderate secretions	Died in 5 days
12.2	1.5	Slight secretions	Recovery
12.2	1.0	Slight secretions	Recovery
23.6	2.0	Moderate secretions	Died in 57 days
23.6	1.0	Moderate secretions	Died in 3 months
23.6	1.0	Slight secretions	Recovery
23.6	0.7	Slight secretions	Recovery
11.3	3.0	Marked secretions	Died in 18 hours
11.3	2.0	Marked secretions	Died in 12 hours
11.3	2.0	Marked secretions	Died in 24 hours
11.3	1.0	Slight secretions	Recovery
29.1	2.5	Marked secretions	Recovery
29.1	2.0	Slight secretions	Recovery
29.1	1.0	Slight secretions	Recovery
71.4	.7	Marked secretions	Died in 48 hours
71.4	.7	Slight secretions	Died in 24 hours
8.2	1.0	Slight secretions	Recovery
8.3	1.0	Slight secretions	Recovery
500H	1.0	Slight secretions	Died in 4 days

After subcutaneous injections, at 3-7 day intervals of increasing quantities of Culture 500, heated for 10 minutes at 60 C., agglutinins were developed in rabbits after 1 month that clumped European and American strains in dilutions of the serum of 1:2000. All the strains mentioned in Table 1 were agglutinated by the same immune rabbit serum diluted 1:1000.

The characteristics of the bacillus of Perez may be summarized as follows: It is gram-negative, grows well in mixed broth cultures, causes a slow fermentation of glucose, decomposes proteins with the formation of mercaptan, produces a characteristic pigment in gelatin, and evokes specific agglutinins in rabbits. These characteristics are adequate to distinguish it from other bacilli, with which it may be associated, such as *B. pyocyaneus*, *B. coli*, *B. mucosus*, *B. proteus*, and *B. bronchisepticus*. The bacillus is readily cultivated from the exudate in ozena, with which condition it is closely associated.

## EXPLANATION OF PLATES

### PLATE 1

FIGS. 1, 5. European strains, 24-hour agar growths.  $\times 1200$ .

FIGS. 2, 3, 4. American strains, 24-hour agar growths.  $\times 1200$ .

### PLATE 2

FIG. 6. Pigment in gelatin culture after 1 month at room temperature, American strain.

FIG. 7. Pigment in gelatin culture after 1 month at room temperature, Vienna strain.

PLATE 1

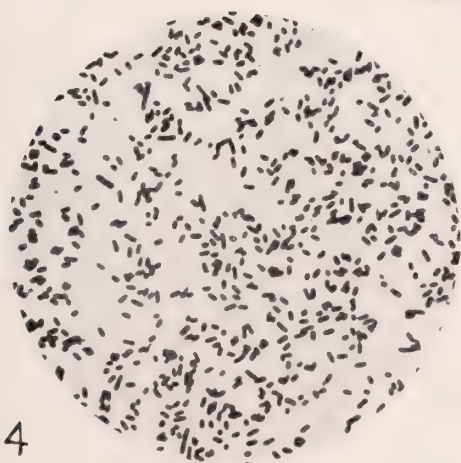
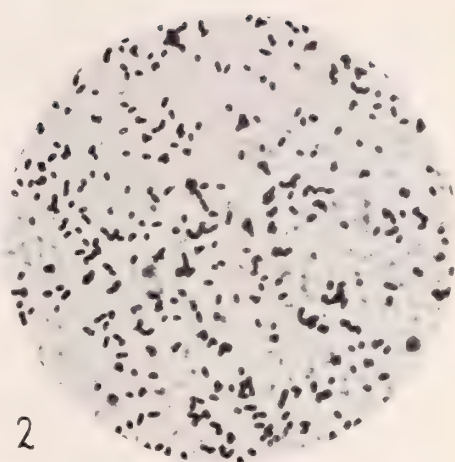
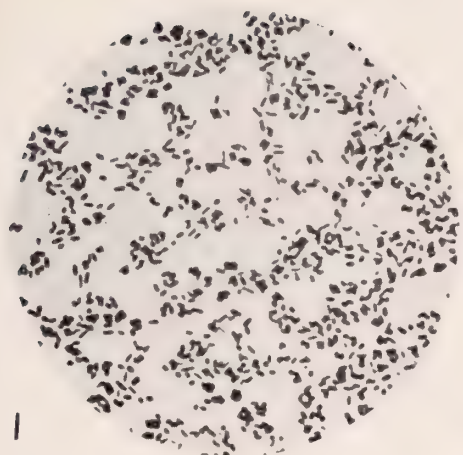




PLATE 2



6



7





# YEAST-LIKE FUNGI OF THE HUMAN INTES- TINAL TRACT \*

PLATES 3-8

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## INTRODUCTION

The fungi as contrasted with the bacteria are relatively unimportant in animal pathology. However, some of the earliest attempts to associate micro-organisms with disease resulted in the discovery of fungi as their causal agents. The fungi causing thrush and ringworm were known and well described before any of the pathogenic bacteria had been isolated. During the last quarter of the 19th century the bacterial diseases have taken much of the time of students of human pathology and it has been only during the last 15 or 20 years that they have again turned their attention to the less important fungous diseases. Interest has been centered largely in those diseases grouped under the general name blastomycoses, so called because the organisms concerned have, at some stage in their life history, budding vegetative cells.

The budding or yeast-like fungi have been so constantly associated with various diseases within recent years and so much confusion exists concerning their life histories and proper botanical position, that it has been thought worth while to make a special study of these organisms from a mycologic standpoint. It was first considered essential to ascertain whether or not the budding fungi were present in the human body, their relative abundance under normal and abnormal conditions and their relation to each other and to the pathogenic blastomycetes isolated by other investigators.

The objects of the present investigation were therefore:

1. To determine the presence or absence and the relative abundance of yeast-like fungi in the normal alimentary tract.
2. To compare the number and kinds isolated from the normal alimentary tract with those found in persons suffering from gastrointestinal disturbances.

\* Received for publication May 24, 1917.

3. To determine the proper botanic position of these yeast-like organisms and to compare them with the pathogenic fungi of similar structure.
4. To investigate the mode of development and the life histories of the fungi secured.
5. To devise a practical scheme of separating species within the group.
6. To determine the fate of yeast-like organisms when ingested.

#### HISTORICAL REVIEW

A brief review of some of the more recent fundamental articles on pathogenic yeasts\* is all that will be attempted at this point. The earlier works have been thoroughly reviewed by Plaut ('03a), Busse ('03), Gedoelst ('02), Guéguen ('04), and Guilliermond ('12), all of whom give excellent bibliographies. Emig ('16) also gives a fairly complete bibliography and reviews some of the more recent publications. Those publications dealing with the taxonomic, physiologic, cultural, and morphologic phases of the subject will be reviewed under the discussion of these items.

Recently Ashford ('15a, b, c) in studying the etiology of sprue, has announced the constant presence of a yeast-like organism in the digestive tract, thus confirming and extending the investigations of Bahr ('14), Kohlbrugge ('01), Le Dantec ('08), and Castellani ('14), all of whom had previously isolated fungi of the *Oidium albicans* type from sprue patients. This disease is present in the southern United States according to Wood ('15), and is of considerable importance in our tropical island territories. It has been suggested by Wood and others that pellagra, having many symptoms in common with sprue, may be caused by a similar organism.

A series of articles by Castellani ('11, '12, '13, '14), Castellani and Low ('13), and Castellani and Chalmers ('13), on the presence of yeast-like organisms of the *Oidium albicans* type in a number of tropical diseases has emphasized the importance of a more intensive study of these fungi. Castellani has recorded the presence of 33 species of *Monilia* as occurring in widely different types of disease. He has also separated the common thrush organism, formerly called *Oidium albicans*, into a number of new species (Castellani '16), on the basis of certain cultural and biochemical reactions.

Recently Simon ('17) has studied an infection of the lungs, of a tuberculous nature, in which he found a yeast-like organism similar to *Oidium albicans*. Birch-Hirschfeld ('75) has recorded a case of the thrush organism involving the lungs, and Castellani ('13) recorded 16 of his species as occurring in broncho-oidiosis. Other investigators have also recorded cases where the thrush fungus was present in the lungs.

The work of Gilchrist and Stokes ('98), Ricketts ('01), Stober ('14), Wade and Bel ('16), and others on blastomycosis in this country will be reviewed in greater detail in another section.

Casagrandi ('98) has made the most complete study of the yeast-like fungi of the alimentary tract. He came to the conclusion that: "(1) In the intes-

\* The term yeasts will be employed in the following pages when referring to the yeast-like organisms under consideration. This shorter term does not imply that the fungi referred to are true yeasts in the narrower sense of the term.

tines of the healthy children there were as many blastomycetes as in those affected with diarrhea; (2) The forms found in both conditions vary from case to case and it cannot be maintained as many wish, that they always belong to *Saccharomyces cerevisiae*, *Saccharomyces ellipsoideus*, etc.; (3) The blastomycetes must, for the present, be regarded as accidental ingredients of the feces, since in the same case observed at different periods they vary so extraordinarily, and since in no case has any one succeeded in discovering a definite form, whether in man or other animals, when these were affected with diarrhea; (4) Up to the present time it has not been possible to bring forward any evidence which will go to show whether the yeasts present in the intestines have any useful or harmful action on the gastro-intestinal functions."

Ashford ('16) has made an extensive study of animal inoculation with his sprue organism both by injection and ingestion. He explains his own negative results in his feeding experiments at an earlier date on the ground that the organism concerned rapidly loses its virulence when kept on artificial laboratory mediums. His positive results were obtained by the use of recently isolated yeasts or those passed through susceptible animals.

#### INVESTIGATION OF YEASTS IN THE HUMAN DIGESTIVE TRACT OBTAINED FROM THE FECES

The series of experiments which follows was planned to determine the presence or absence and relative number of yeast-like organisms in the human alimentary tract, and to discover any relation which might exist between their presence and gastro-intestinal disturbances of various kinds. They were intended to extend and supplement the investigations of Ashford and others. In addition, it was hoped that a study of such organisms as were isolated would furnish a means of distinguishing the pathogenic yeasts from those harmless ones accidentally present but frequently occurring in the feces.

#### METHODS

*Mediums for Isolation.*—The fact that the feces contain such a large number of bacteria, as compared with the fungi, has made the isolation of the latter group very difficult when using the ordinary culture mediums and standard plating methods. For this reason, a medium which restricted the growth of the bacteria had to be employed. The most useful medium for this purpose is undoubtedly Sabouraud's agar, as suggested by Ashford ('15c). Since the method of preparation has been somewhat modified and since the standard textbooks on bacteriologic methods do not include the formula for this valuable medium the following account is included:

Agar .....	20 grams
Peptone .....	10 grams
Glucose .....	40 grams
Water .....	1 liter

The agar, peptone, and water are mixed and dissolved in the usual way. The medium is then titrated and rendered +2 acid with normal hydrochloric acid. Without further heating, the sugar is added and the medium tubed in previously sterilized tubes. Care is necessary in sterilizing since the high acidity of the

medium may prevent solidification. Ten minutes at 5 pounds pressure is usually sufficient. This is better than discontinuous sterilization, since reheating the agar seems to destroy its power of solidifying under these conditions. The high acidity of the medium prevents the bacteria of the feces from developing rapidly.

*Collecting and Plating Samples.*—Since yeasts are present in fairly large numbers in the air and on unsterilized objects, it was necessary to collect the samples with as little contamination as possible. For this purpose small vials,  $20 \times 70$  mm., with wooden spatulas firmly fixed in the cork and extending almost to the bottom of the vial were prepared. These could be sterilized in the hot air sterilizer with the cork and spatula in place. By means of the spatula a small portion of the sample to be studied was introduced into the vial, without contact with external sources of contamination. In this condition the vial could be shipped some distance without danger of contamination and with little change in the water content of the sample.

The regular dilution and poured plate method was found unsatisfactory for the primary cultivation of the yeasts and their separation from the bacteria. This was probably due to the fact that the yeasts develop rapidly only on the surface of the plate and few are left on the surface under these conditions. Another method was therefore employed in most of the isolations. This is a modification of the method used by Ashford.

Three plates of the acid glucose agar were poured and allowed to harden. A stiff platinum needle was then sterilized and thrust into the sample. The agar was touched lightly at points about 4 mm. apart in a line across the plate. This process was repeated, each series of contacts forming a row across the plate, 10-20 contacts being the usual number in each row. There were usually about 20 rows on each plate, thus giving 200-400 contacts. This procedure was modified somewhat when a large or very small number of yeasts was expected, or where the consistence of the feces made a change necessary.

While no exact quantitative results were expected by the use of this method some interesting comparative results were obtained. A sample which contained a relatively large number of yeasts would develop 20-200 yeast colonies on each plate, while in those in which only the normal number was present, only 2 or 3 colonies appeared on each plate, or 5 or 6 colonies would appear on 1 plate and none on the other two. This striking difference was especially clear in the feeding experiments where the number would jump from less than 1% of the contacts before feeding to 80 or 95% immediately after feeding, and then back again to 1% or less a few days later. The dilution method was used in a number of cases as a check on this contact method with the result that the latter was found to be even more accurate than was expected. There is no question but that it gives accurate comparative results when carefully carried out.

The yeast colonies which were developed on the medium were usually pearl-white or pink in color and could be distinguished from the usually clear bacterial colonies. However, every distinctive colony on a plate was examined in order not to overlook a possible unusual yeast growth. An objection that might be urged against this method is the possibility of yeasts existing in the feces as sensitive to acid conditions as are the bacteria. In order to test this, a large number of yeasts were obtained from various sources and each was plated on agar directly, or mixed with the feces and then plated. In no case did the yeast fail to develop. All the common species of *Saccharomyces*, *Mycoderma*, *Torula*, *Oidium*, and *Cryptococcus* were tried. Also undetermined yeast-like fungi from fruits, fresh yeast-cake, air, soil, stomach contents, etc., were used with like results.



Table 1 gives a numerical summary of the results obtained from the study of samples taken from 175 persons by the methods described.

TABLE 1  
SUMMARY OF ISOLATIONS OF YEASTS FROM 175 PERSONS EXAMINED

Total number of persons from whom samples were obtained .....	175
Total number of samples plated.....	229*
Total number of samples yielding yeasts.....	85
Total number of yeast isolations.....	113†
Percentage of samples yielding yeasts.....	37
Total number of persons without gastro-intestinal disturbances.....	98
Total number of diarrheal cases (exclusive of sprue and pellagra cases).....	31
Total number of uncertain history .....	27
Total number of pellagra cases.....	18
Total number of sprue cases.....	1
Total number of yeast isolations from 98 normal persons.....	46‡
Percentage of normal persons yielding yeasts.....	47
Total number of yeast isolations from 31 diarrheal patients.....	13
Percentage of diarrheal patients yielding yeasts.....	45
Total number of yeast isolations from 18 pellagra patients.....	4
Percentage of pellagra cases yielding yeasts.....	22
Total number of yeast isolations from 1 sprue patient based on 8 trials.....	8
Percentage of yeast isolations in sprue patient (8 trials).....	100
Total number of yeast isolations from 27 unknowns .....	14
Percentage of yeast isolations from unknowns .....	52§
Number of healthy persons showing large numbers of yeasts (10% or more of contacts) on first trial .....	9
Number of diarrheal patients showing large numbers of yeasts on first trial.....	0
Percentage of sprue patients showing large numbers of yeasts.....	100
Number of pellagra patients showing large numbers of yeasts.....	1

\* In several cases a number of samples were taken from the same person at different dates.

† Two or 3 different species frequently occurred on the same plate from 1 person.

‡ A plate containing several species was regarded as a single isolation in this and the following results in the table.

§ The high percentage here is due to the fact that some of these isolations were made by others who reported only successful results. In cases which I actually recorded the percentage approached that of the normal persons.

|| Percentage results are given here since only a single case of sprue was included, but a number of trials proved the uniform presence of yeasts in the feces.

## DISCUSSION OF RESULTS

The percentage of positive results recorded in Table 1, based on the total number of isolations, is undoubtedly lower than it should be because, in a number of cases, an *Oidium lactis* type of fungus developed so rapidly as to prevent the growth of yeasts had they been present. In other cases, especially in diarrheal stools, certain slime-forming bacteria developed in spite of the acidity of the medium. Furthermore, the small quantity of feces used for plating would naturally reduce the number of positive results. There is little question but that yeasts are present in small numbers in practically all human feces.

The percentage of positive results from healthy persons and from those with diarrhea is approximately the same. The sprue patient, in 8 recorded trials, yielded over 50% of colonies in every plating. Thus the fact that the results were positive in this case is not as significant as finding a very large number in every trial. The pellagra

cases yielded less than the normal number of positive results. However, *Oidium lactis* was present in several of the pellagra stools and had an unusual chance to develop before plating on account of the long time in transit from the point of collection (Spartanburg, S. C.). Only 1 of the pellagra patients showed a large number of yeasts in the first trial. Failure to secure more samples from this person rendered the results incomplete.

The results of Ashford and those I recorded in a sprue patient indicate that the constant presence of large numbers of yeasts in the stools is more significant than the presence of a few in each sample. For this reason particular attention was given to those persons in whom a large number of yeasts was found in the first plating, and a special effort was made in such cases to secure samples at intervals from these same persons. It will be shown from the feeding experiments that a comparatively small amount of yeast material ingested with the food will cause a very decided increase in the number found in the feces. Therefore, the presence of a large number from 1 sample could be accounted for by the fact that the person had eaten fruit or other food containing an abundance of yeasts. If repeated trials showed a constant large number some other cause would have to be sought. In Table 1 it is seen that 9 healthy persons gave large numbers of yeasts on the 1st trial. Subsequent samples from these same persons showed, on the 2nd trial, only 2 giving large numbers, and on the 3rd trial only 1 continued to give positive results. This last case was that of a woman about 65 years old in the Kankakee State Hospital for the Insane. A record of this patient showed that she had no gastro-intestinal disorder but was in a very weak condition during the course of the trials. After the 5th positive sample had been obtained she died from a cerebral hemorrhage. She was given the ordinary diet.

Table 1 shows that none of the diarrheal patients gave large numbers of yeasts, and that in only 1 pellagra patient was there an unusual number present. These results indicate that in the sprue case the constant presence of a large number of yeasts in the feces has some special significance. From the fact that in sprue the stools are decidedly acid, and that acidity favors the development of such organisms as those found in sprue patients, one might argue that their presence is only indicative of abnormal conditions arising from other causes. Thus the thrush organism develops in the mouth of infants before the secretions render it alkaline, and a number of investigators have stated

that the appearance of the thrush organism in the mucous membrane of adults indicates an acid condition. The presence of abundant yeasts might, therefore, be an indication of abnormal conditions in the intestinal tract. If this hypothesis were true one would expect to find large numbers of yeast-like fungi constantly present in other types of intestinal disorders which give similar conditions to that of sprue or thrush. The results obtained in this investigation show that comparatively few diarrheal patients yield large numbers of yeasts, and indicate that some other explanation of the large numbers found in sprue cases is necessary.

The geographic distribution of the persons studied was found to be of no particular significance. Persons from the southern states gave no different results from those in the north. A large number of samples were obtained from the Kankakee State Hospital for the Insane. These samples were taken at different times extending over a period of several weeks, thus securing them under varying conditions of diet. The samples from pellagra cases were obtained from the United States Pellagra Hospital, at Spartanburg, S. C. A number of persons from Urbana and Champaign also furnished material for study. An epidemic of gastro-intestinal trouble at the Lincoln State School and Colony, at Lincoln, Illinois, furnished an exceptional opportunity for studying diarrheal cases. Local physicians kindly secured a number of miscellaneous cases of intestinal disturbances.

The sprue case recorded is of special interest since it is one of the few studied in temperate climates from an etiologic standpoint. The person from whom the isolations were made had lived for 2 years in Porto Rico (1912-1914) where it is supposed she contracted the disease. She then came to Urbana and has lived here since, except that the summer of 1915 was also spent in Porto Rico. The present tests were made during the fall of 1916.

As mentioned, the diet of the person could evidently influence the number of yeasts present at any one time. It was not possible, however, to make a detailed study of the diet of the persons examined and no attempt was made to control this factor except to secure samples from a large number of persons under different conditions of diet and at various seasons of the year.

Summarizing the results obtained from 175 persons, it is evident that yeast-like organisms are present only in relatively small numbers in healthy persons; that ordinary gastro-intestinal disorders have no relation to the yeast flora of the intestinal tract; that the constant presence of large numbers of yeasts is not met with in healthy or diseased persons under ordinary conditions; and that in the single sprue patient examined, a species of yeast was constantly present in large numbers in all samples plated.

## MORPHOLOGIC, PHYSIOLOGIC, AND CULTURAL STUDIES OF ORGANISMS ISOLATED

The 113 organisms isolated were grown on a number of mediums in order to make a preliminary separation into groups on the basis of striking differences. For comparison, the determined organisms given in Table 2 were obtained from various sources. In addition to these a number of undetermined species isolated from the gastric residua of normal persons was furnished by Mr. Max Levine of Iowa State College; organisms which are of special interest on account of their source. Several cultures of species of *Mycoderma*, *Torula*, and *Monilia*, indicated by number only, were received from different laboratories. Cultures isolated from different fruits during the course of this study were also used for comparison, as was also *Sclerotinia* (*Monilia*) *cinerea* isolated from plum. Dr. Simon also kindly sent me the organism described by him as causing a tuberculous condition of the lungs. *Blastomyces dermatitidis* was obtained from the Memorial Institute of Infectious Diseases in Chicago and from the American Museum of Natural History. Thus, in all, over 160 cultures were used in the preliminary study of the group of budding fungi. While only a limited number of these were studied in detail, a series of comparative observations was made on the entire series. As the result of a preliminary study the organisms isolated were separated into a number of large groups which are described.

Variation in color furnished the first basis of separation. There appeared in a large number of the original plates pink or red colonies of yeasts which made gelatinous watery growths and appeared very similar in gross characters. Isolations of these were not attempted except in about half the first samples tested, since it was early decided that most were of the same species. Whenever a variation in these pink colonies was observed, such as shade of color, type of growth, or shape of cells on microscopic examination, the form was isolated and saved for future comparison. Four types of red or pink yeasts were thus obtained which will be referred to only as Types 1-4 since this paper includes a specific study of only 1 of these.

The 2nd group, on the basis of color, included those of a chalky-white appearance on agar slant cultures. These constituted the largest percentage of yeasts isolated and it is with this group that I am now especially concerned.

The 3rd group develops a yellow, golden, or gray colony on agar plates. This is a miscellaneous group for the inclusion of all the odd forms not included in the first 2 groups. It is especially important, however, since many of the so-called pathogenic yeasts are of this type. Only 2 isolations of this type were made but several of Levine's yeasts from normal stomach contents belong here. Most of the yeasts of this type liquefy gelatin and develop more or less of a slimy growth on most mediums. These 3 groups will be designated as the 'pink', 'white', and 'golden' groups.

The white group, on account of the number of forms isolated and their similarity to most of the important pathogenic yeasts described in the literature as '*Monilia*' and '*Oidium*' species, were made the object of special study. This group could be subdivided into 2 very distinct subgroups: (1) those which form a slightly to decidedly heaped, glistening growth, especially in young slant cultures, and (2) those which form a spreading, dull-white growth on agar slants and usually produce a heavy, dry pellicle within 48 hours on liquid mediums. The latter type will be designated the '*Mycoderma*' type since this characteristic growth is the basis of the formation of this genus according to Hansen, Will, and others.



TABLE 2  
LIST OF FUNGI USED FOR COMPARATIVE STUDY

Number	Name of fungus*	Source and Remarks
1	<i>Saccharomyces cerevisiae</i> .....	Parke Davis & Co., Detroit
2	<i>Saccharomyces glutinis</i> .....	Parke Davis & Co., Detroit
3	<i>Monilia candida</i> Bon.....	Parke Davis & Co., Detroit
4	<i>Monilia candida</i> Bon.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
5	<i>Monilia humicola</i> Oudem.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
6	<i>Monilia sitophila</i> (Mont.) Sacc.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
7	<i>Oidium humi</i> Maze .....	Centralstelle für Pilzkulturen, Amsterdam, Holland
8	<i>Oidium lactis</i> Fresenius.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
9	<i>Oidium pullans</i> Lind.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
10	<i>Saccharomyces hominis</i> Busse.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
11	<i>Saccharomyces Pastorianus</i> Hansen....	Centralstelle für Pilzkulturen, Amsterdam, Holland
12	<i>Saccharomyces glutinis</i> (Fres) Cohn....	Centralstelle für Pilzkulturen, Amsterdam, Holland
13	<i>Saccharomyces ellipsoideus</i> (I) Han...	Centralstelle für Pilzkulturen, Amsterdam, Holland
14	<i>Saccharomyces cerevisiae</i> Hansen.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
15	<i>Saccharomyces anomalus</i> Hansen.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
16	<i>Saccharomyces</i> (pathogenic) Binot.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
17	<i>Saccharomyces</i> (pathogenic) Curtis.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
18	<i>Saccharomyces</i> (pathogenic) Foulerton..	Centralstelle für Pilzkulturen, Amsterdam, Holland
19	<i>Schizosaccharomyces Pombe</i> Lindner...	Centralstelle für Pilzkulturen, Amsterdam, Holland
20	<i>Schizosaccharomyces octosporus</i> Beyer- inck .....	Centralstelle für Pilzkulturen, Amsterdam, Holland
21	<i>Torula glutinis</i> (Conn) Pringsh. et Bi- lersky .....	Centralstelle für Pilzkulturen, Amsterdam, Holland
22	<i>Torula humicola</i> Daczewska .....	Centralstelle für Pilzkulturen, Amsterdam, Holland
23	<i>Torula rubra</i> Schimon .....	Centralstelle für Pilzkulturen, Amsterdam, Holland
24	<i>Willia belgica</i> Lindner.....	Centralstelle für Pilzkulturen, Amsterdam, Holland
25	<i>Blastomyces dermatitidis</i> Gilchrist and Stokes .....	American Museum of Natural History
26	<i>Blastomyces dermatitidis</i> Gilchrist and Stokes .....	Memorial Institute of Infectious Diseases, Chicago
27	<i>Oidium albicans</i> Ch. Robin.....	London, England, H. D. Singer, Kankakee State Hospital

\* The names of the fungi and the authors of the species here listed are those used by the person sending the cultures.

After the separation of the cultures on the basis of the gross characters, a morphologic study of a large number of these forms was undertaken. The criterion used in selecting these fungi from others which frequently occurred on the same plate was their 'yeast-like' appearance. By this is meant the formation in young cultures of single cells which reproduce by budding, in other words, unicellular, budding organisms. Before going into more detailed description of morphologic characters a discussion of the significance of the budding process will be given.



## THE SIGNIFICANCE OF BUDDING

The production of a new cell or a new individual plant by budding is an entirely different process from the production of a new cell, conidium, or spore by septation or abjunction. In the budding process a protuberance is formed at some point on the parent cell. This swells and increases in size in the distal portion but not at the junction. This results in a narrow neck which remains narrow during the further growth of the cell. When the daughter cell has reached a certain size the protoplasm at the neck is separated and a wall is formed between the end of this cell and the portion of the original cell from which the bud arose. This results in 2 distinct and separate individuals, and the daughter cell is very easily detached from the parent. In the process of septation, on the other hand, the parent cell elongates and the protoplasm is separated by a cross wall. If the fungus has a hyphal development this usually takes place at the end of the hypha, that is, the growth is apical. If a branch is formed a protuberance appears as in a bud, but there is no constriction or only a slight constriction at the point of attachment to the parent hypha. If budding is regarded as a type of reproduction comparable to the formation of conidia a difference may also be seen here in most cases, in that usually the end of the sporophore elongates and the conidia are cut off by septation as in the formation of a new cell. The cell which is cut off to form the conidium may enlarge, become rounded, or change its shape in various ways. In some cases a condition falsely simulating budding seems to occur, in that near the end of the elongated sporophore there appears a sinus which gradually narrowing squeezes off the spore. But in this case the conidium is from the first as broad as the parent hypha. In some of the lower hyphomycetes there are conditions of conidial formation which closely approach true budding. In these cases the resulting conidia when mature are definite in form and size, and, on germination, produce true germ tubes. In the case of the yeasts the buds are in no sense conidia or spores of the parent plant. Rather they are new individuals ready to grow and carry on all the necessary metabolic processes of the organism. On germination, conidia or spores send out true germ tubes by the further growth of which a mycelium is formed. In the budding process, on the other hand, the new cells formed, on further growth, produce new buds and not germ tubes.

It is recognized that the fungi which develop a true mycelium, such as Exoascaceae and Ustilaginales may have budding stages in their life histories. Budding is not rare in most of the larger orders of fungi, and may appear under abnormal conditions in many forms (de Bary '87). It is not maintained, on the other hand, that the yeast-like fungi described in this article never form a septate mycelium. But it is asserted that there exists a group of fungi which, under a very wide range of cultural conditions, rarely form septate hyphae, but on the contrary produce new individual, unicellular plants almost exclusively through the process of budding.

## GENERAL MORPHOLOGY

Microscopic examination of all the cultures revealed a wide range of morphologic characters. The only character all forms had in common was that of budding. In young cultures, in all cases, the only form was the budding yeast-like cells. These usually contained more or less distinct vacuoles, although such were frequently absent. One or more refractive granules was also evident in the vacuole or cytoplasm. In old cultures a decided change was usually evident in the structure and form of the cells. In many cases, the

cells had elongated, and, remaining attached to each other, formed a hyphal thread constricted at the ends of the cells. These 'articles' were easily broken apart with the result that elongated cells were scattered among the shorter budding cells. In these old cultures 'giant cells', frequently 10 times the diameter of the normal cells, were present. The cell contents also changed, usually 1 or more large 'oil' globules appearing in each cell and frequently completely filling it. In other cases no elongated or giant cells were found and the individual cells were very small and showed no budding.

The forms which these yeast-like fungi assume are various. Figure 1 represents, diagrammatically, a series of forms on which the terminology in the plates is based.

The formation of a series of elongated cells does not necessarily imply that the yeast concerned has given up the budding habit in favor of hyphal formation and septation. In the majority of cases such series of cells are formed by the apical budding of each cell in turn and the subsequent elongation of the members of this series of cells. In certain species, however, and under conditions which will be explained in more detail, the cells pass over into elongated mycelial threads which form true septa (Plate 3, Fig. 13) just as in cell division in the other fungi. This condition, however, is rare and even here

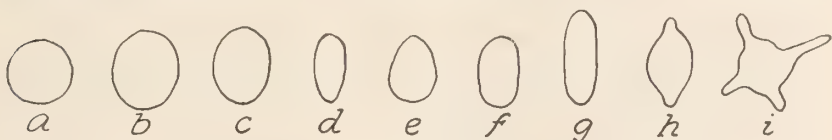


Fig. 1. Forms of Yeast-Like Fungi: a, round; b, oval; c, elliptical; d, narrowly elliptical; e, ovate; f, oblong; g, elongated; h, limoniform apiculate; i, amoeboid.

the individual cells of the mycelium send out true buds, which, in turn, bud in the same manner as the cells in a young culture. A large number of species do not form septate hyphae under any condition so far discovered. There are, therefore, 3 distinct morphologic groups in the forms studied: (1) those which have only round or oval cells, (2) those which may form elongated cells but do not produce septate hyphae, and (3) those which form elongated cells which may pass over into septate hyphae. There is not a sharp distinction between the first 2 groups but these are fundamentally different from the last.

In all the forms studied, even when a septate mycelium was developed, there was never a tendency to form a dry, aerial mycelium, such as is produced by the majority of fungi when in culture. The yeasts were grown on or in a very large number of mediums under a variety of conditions as to moisture and temperature, but never was there an approach to aerial mycelial production. This is of fundamental importance in distinguishing these forms from such types as *Oidium lactis*, *Monilia cinerea*, and *Blastomyces dermatitidis*.

#### THE BASIS OF GENERIC AND SPECIFIC SEPARATION WITHIN THE GROUP

The forms isolated from the intestinal tract, when tested for endospore formation by various standard methods, were found to be, with 2 exceptions, asporogenic species. Since the majority of pathogenic fungi and a very large number of the species of yeast-like organisms in nature are of this type, the following discussion will deal exclusively with the asporogenic forms. Since they have no sexual or 'perfect' stage they are naturally included in *Fungi imperfecti*.

*Generic Separation.*—In the preceding section it was stated that a distinct morphologic difference exists between the forms which never produce new cells by septation and those which may show septation under proper conditions. This fundamental difference has been recognized by a number of writers. Cao ('00), on the basis of the development of a villous growth in gelatin-stab cultures and the formation of elongated cells, wished to place all such forms in the genus *Oidium*. He described a large number of such fungi using numbers only to designate his 'species.' He did not distinguish between those which form elongated cells without septation and those which produce septa. Will ('03-'08), in several instances in his articles on "Sprosspilze ohne Sporenbildung" called attention to these 2 types. Geiger ('10) also differentiates clearly between the 3 groups enumerated. He believed that the forms having septate hyphae should be placed in the genus *Monilia* on the ground that *Monilia candida* is of this type. He erects a new genus, *Pseudomonilia*, to include 4 species having elongated narrow cells of a hyphal nature, but without septa. The forms producing septate mycelium under certain circumstances but usually forming budding cells are included, by most authors, under either the genus *Monilia* or *Oidium*. The latter genus is, as a rule, used to designate forms of the *Oidium lactis* type. The species which do not form these elongated septate hyphae are usually placed in the genus *Torula* if they do not form a pellicle in liquid mediums, while if such is formed, they are called *Mycoderma*.

It is recognized that there may occur conditions under which the non-septate might pass over into the septate type. But a series of observations on a number of forms which produce elongated cells but do not form septa tends to show that the character is constant.

We have, therefore, a group of the Fungi imperfecti which is distinguished by the fact that, with the conditions under which they grow and under all the usual conditions of laboratory culture, reproduce by budding exclusively and do not form a dry, aerial mycelium. This group in turn may be separated into 2 distinct morphologic groups on the basis of septation or nonseptation. These 2 groups should be given generic rank and such a classification is to be proposed.

The formation of elongated, radiating, thread-like filaments in gelatin-stab cultures does not prove that septate hyphae are formed. Hanging drop cultures which are allowed to develop several days are most useful in showing this character. The filaments extend from the drop, in the case of solid mediums, into the water of condensation on the cover glass, and may be examined even under an oil immersion objective. By watching these for an hour or so the formation of septa may be observed if it occurs. Various mediums may be used for hanging drop cultures but a most satisfactory one is 0.5% beerwort agar solution. A tube containing such a solution is melted and a few yeast cells on the end of a needle are introduced. The tube is then shaken and a portion drawn up into a sterile glass tube with a fine drawn end. The drops are then placed on a sterile cover glass and inverted on a ring in the usual manner.

*The Basis of Specific Classification.*—The basis of specific differentiation in most microscopic organisms, aside from the bacteria, is morphologic. In the fungi the character of the mycelial growth, the form, size, marking, and arrangement of the reproductive bodies, whether sexual or asexual, are used for separating genera and species. When any of these characters vary within the species, the limits of variation are not wide, and may be easily determined by biometric methods. Frequently internal structures, thickness of spore walls,

etc., aid in the separation of species. In the yeast-like organisms under consideration there are certain difficulties encountered in attempting to separate the species by the various characters given. The yeasts are primarily unicellular organisms, even when an apparent mycelium is formed. These individual cells tend to assume a certain fairly uniform shape under a definite set of conditions (Fig. 1). When 2 species are examined side by side under the microscope it usually is easy to see that they are different. But when a number of cells of 1 of these species are measured a wide variation is observed, and when compared with the other species these variations cause the measurements to overlap to such an extent that it is impossible to distinguish, from description, which of the 2 is under consideration. A very large number of measurements possibly would reveal a constant difference between the 2 species, but the necessary measurements would be too laborious and uncertain for practical use. It is highly desirable that a system of species differentiation be devised which is practical for the average laboratory worker.

The difficulties of differentiating species arising from this great variation in the size and form of the cells has been recognized by such workers as Hansen, Will, and Geiger. They maintain, as I do, that the morphologic characters have little analytic value when taken alone.

An attempt was made to use internal structures such as size, number, and position of granules, the relative refraction of the protoplasm, the size, number, and location of the vacuoles, etc., as a basis for separating species. It was found, however, that these internal structures varied according to the age of the cell and the medium in which it was placed. At one stage a small vacuole with a single dancing particle within it was present; a few hours later this vacuole would be much larger and there might be several dancing particles in it, or the vacuole might seem to disappear entirely, due, probably, to the change in the refractive index of the solution. Thus no satisfactory means of separation could be found by a study of internal structure.

The form of the cells is fairly constant in some species. For example, *Saccharomyces cerevisiae* differs from *Saccharomyces pastorianus* in a constant manner, and one would have no difficulty in separating these 2 species under the microscope. But the number of possible forms the yeasts can assume is very limited, as is indicated by Figure 1. Furthermore, a single species may have cells of all of these forms in a single field of the microscope (Plate 3, Fig. 10). This is especially true of the 'wild' yeasts under consideration.

The manner of budding is constant in many species. If a given species is cultivated under a fairly wide range of conditions the buds may uniformly appear at some definite point on the cell. But here again there are only a few possibilities of differences. A large number of species may bud in the same manner and again there are forms which may vary in their manner of budding under varying conditions. The tendency to form elongated cells of a hyphal character is constant in certain species, but is present in a large number of species.

All of the characters mentioned, when used alone or in combination, are of value in the description of a species; but the general statement can be made that no constant morphologic character or combination of characters can be found which is uniformly reliable in differentiating single species, on account of the great tendency to vary among species and the limitation of possibilities in such simple organisms.

From this discussion it is evident that morphologic characters must be used in connection with biochemical properties and cultural peculiarities in the differentiation of asporogenic yeast-like species in the same manner in which the



bacteriologist makes use of these properties. Will ('03-'15) has made an exhaustive study of the asporogenic yeasts, using biochemical properties and cultural characters as a means of differentiating the 'forms' obtained. Geiger ('10) also uses Will's methods in his study of the species of the genus *Pseudomonilia*. The assimilation of different sugars, alcohols and organic acids, the production of alcohol and acids, the resistance to alcohol, etc., are some of the characters determined for each form studied. Both authors also emphasize the importance of the use of 'giant colonies.'

Lutz and Guéguen ('01) have proposed a classification based on morphologic and cultural characters. They wish to establish a standard method of procedure to use with all hyphomycetes such as the bacteriologists employ for differentiating bacterial species. They recommend the employment of a synthetic medium (Raulin's solution\*) with and without various sugars, the addition of other carbohydrates, glycerin, etc. They also employ milk, potato, and carrot slants and egg albumin.

The complicated procedure recommended by Will cannot be undertaken in ordinary routine work on account of the complex chemical analysis employed, and the time necessary to complete the study of a species. This is especially true where pathogenic yeasts are to be studied, since the method employed by Will requires at least 3 months for complete observation. The procedure recommended by Lutz and Guéguen has much in its favor, but does not include several of the most important differentiating mediums. The yeast water employed by Will and others is more satisfactory as a basic solution although its chemical composition is not as definite as that of the synthetic medium of Raulin.

Variations in cultural and physiologic characters are encountered in the yeasts, even to a greater extent than in the bacteria. This is especially true in streak cultures on solid mediums such as agars, carrot and potato slants. Variation in fermentation of sugars and in acid-production is also frequently encountered. Ashford ('15d) has recorded the fermentation of galactose and sucrose by his sprue organism at one time, while at another time it failed to produce gas in these sugars.

In studying the organisms isolated a large number of different mediums were used and the cultural, physiologic, and biochemical properties of these yeasts were studied under a wide range of conditions. The object has been to secure differential methods which are based on procedure ordinarily employed in bacteriologic or mycologic laboratories. At the same time it is realized that additional information along biochemical lines aids in a surer differentiation and gives more insight into the complicated life processes of the organisms concerned. The mediums which have been found most useful are discussed in detail in the next section.

Agglutination and precipitation tests were not attempted. There is a possibility that some satisfactory basis for specific differentiation may be obtained by the use of these properties, though the results obtained by various investigators thus far have been disappointing.

\* Raulin's solution:

Distilled water .....	1,500	gm.
Sucrose .....	70	gm.
Neutral potassium tartrate .....	6.5	gm.
Ammonium nitrate .....	4.5	gm.
Potassium phosphate .....	.6	gm.
Magnesium carbonate .....	.4	gm.
Potassium sulphate .....	.25	gm.
Ferric sulphate .....	.07	gm.
Zinc sulphate .....	.07	gm.
Potassium silicate .....	.07	gm.



## MEDIUMS EMPLOYED FOR SPECIFIC DIFFERENTIATION

*Sugar Medium.*—Hansen has separated the genus *Saccharomyces* into 6 subgroups based on their fermentative action toward 4 sugars: glucose, sucrose, maltose, and lactose. Guilliermond ('12) has also based his preliminary separation of the genus on these reactions. Castellani ('16) has used a much larger number of sugars and other carbohydrates in differentiating his '*Monilia*' species and he emphasizes the importance of acid-formation in the mediums. He goes to the extreme in this direction in that he separates species in the genus on the basis of slight differences in reaction with sugars alone. Ashford ('15d), criticizes Castellani and doubts the value of the use of sugar mediums alone. He recognizes the value of such mediums, however, but insists that the results are to be used only in connection with other differentiating characters. Castellani's results should be confirmed by other investigators or a more detailed account of the methods employed by him should be given before his conclusions are accepted. Geiger ('10) has shown that in various sugar mediums, during 3 months, with different yeasts, there may be a decrease, then an increase in acidity, or an increase followed by a decrease, or a steady increase or decrease. Recording the length of time the cultures are grown and the temperature at which they are inoculated is of great importance.

The sugars selected in the present study after preliminary experiments were glucose, sucrose, lactose, maltose, galactose, levulose, and raffinose. Dextrin was used for studies in acid-production and other cultural characters. None of the yeasts investigated produced gas with either lactose or raffinose, but the former was included because it has been generally used by other investigators and the latter because it is a trisaccharid. The importance of using very pure sugars cannot be overemphasized, and the lack of this precaution undoubtedly accounts for the many positive results reported by some investigators for sugars that are not ordinarily fermented.

Fermentation tubes of the Smith type were used in all cases to determine the presence or absence of gas production. The Durham tube-within-a-tube method was tried but proved unsatisfactory. In the determination of acidity a 200 c.c. flask with 100 c.c. of medium was used. The cotton plugs in the flasks were covered with waxed paper held by a rubber band in order to prevent excessive evaporation. The large amount of medium was necessary to allow for the 3 or 4 series of titrations made. The formation of rings, films, and other cultural characters could also be better studied in flasks.

The basic substance employed in the sugar medium may have an important bearing on the nature of the reaction obtained. Since yeast water forms an ideal medium for the growth of all yeasts studied, and since it has been commonly employed by Will and others working on this group of asporogenic yeasts, it was thought best to employ it as the main basic solution. The results obtained from the use of this medium were not different in most respects from those obtained by the use of 'nutrient broth' as the basic substance, except in the acidity tests. It is important that some standard solution be selected for the use of all investigators, such as is employed by all bacteriologists. The bacteriologic mediums are not entirely satisfactory and are more complex than is necessary for the cultivation of the yeast-like organisms. Since the yeast water solution is easily made and the material used in its composition is generally available, it is recommended that it be made the standard for future investigations on this group.

The yeasts used for inoculation were taken from young cultures on glucose agar slants. The tubes and flasks were run in duplicate with a control set

uninoculated. Another set of yeast water alone was inoculated with the same organisms. One series was kept at 35-36 C. and another at 25-27 C. Before recording the results with any yeasts several trials were made in order to eliminate chance variation.

The variations in fermentation of sugars recorded by Ashford in the case of his sprue organism were not met with when vigorous young cultures were used. On several occasions when cultures of different ages were taken at random variation was noted. The cause of this variation was not determined, but there is no doubt but that it does occur and should be guarded against.

*Litmus Milk.*—This medium is useful for separating the yeasts into 3 distinct groups: (1) those which render the milk more alkaline, (2) those which produce no marked change, and (3) those which produce acidity. No yeast studied coagulated milk within 2 weeks, and very few produced acidity. Practically all the forms isolated from feces belonged to the first 2 groups. By acidifying the litmus until it has a strong violet color the change to the alkaline condition is very marked. The tubes were incubated at 25-27 C. and 35-36 C. and were observed at frequent intervals.

*Gelatin Mediums.*—Gelatin-stab cultures are especially useful in determining whether or not the yeast studied produces elongated cells. Yeast water, beerwort, and nutrient broth gelatins were most commonly employed. The beerwort gelatin is most satisfactory for growing giant colonies since it is easily prepared. The stab cultures are most satisfactorily observed in yeast-water gelatin since gas-forming yeast produce bubbles in beerwort gelatin and break up the mediums. The type of growth in gelatin depends to a certain extent on the consistency of the medium and care should be taken to secure a firm gelatin.

A few of the yeasts studied liquefied gelatin while the great majority did not. There was a decided variation in several species which were at one time liquefying, at another not. Such variations may account for the difference in the description given of *Oidium albicans* by several authors. Frequently old cultures were found to begin liquefaction after they were left several weeks undisturbed. Such liquefaction is probably not due to the activity of the living cells, and should not be regarded as what is ordinarily spoken of as liquefaction.

Three general types of growth in gelatin are observed among the white, nonliquefying group. In the 1st type there is a more or less uniform, straight, white line following the needle puncture. This is filiform at first but may later become decidedly nodose. Practically all yeasts take this form during the first few days, and then pass over into 1 of the 2 types to be described. The 2nd type has a villous form of growth. Within this type there is a wide variation from forms with individual filaments extending outward at right angles from the axis, like the bristles on a brush, to those having branched bushy filaments. The villous condition may develop early, as in certain *Mycoderma* types, or it may not appear until the culture is a week old. The denseness of the radiating hyphae, their length, and degree of branching is usually characteristic. Under the hand lens these radiating threads often appear moniliform due to the groups of buds at the ends of the cells of the hyphae. The 3rd type is really a modification of the 2nd, but on account of its importance in distinguishing forms of the *Oidium albicans* type, it is described separately. At first the growth is filiform; later it becomes somewhat nodose. In about 5 or 6 days after inoculation there appear radiating hyphal elements near the surface of the medium. These may extend to a depth of one-half inch below the surface or may not appear at all. Later from scattered points along the nodose line, fine, individual, bushy growths appear. There may be only 4 or

5 of these along the entire puncture or they may be so close together that the culture approaches the 2nd type of growth. Occasionally some cultures do not develop these at all. For this reason several cultures were usually made at one time. In other cases instead of the bushy growth appearing, there were developed a number of root-like radiations resembling the secondary roots arising from the tap root. To this group belong *Oidium albicans*, the sprue organism, and 1 or 2 others which will be described. Plaut ('03b) figures a typical growth of this kind. In the study of the white and golden types described, 52 cultures were tested in gelatin stabs. Of these 30 showed a villous growth, while 22 were nodose or echinulate. In a study of 22 named cultural yeasts, 11 produced a villous growth while the same number did not.

*Sugar-Free Mediums.*—Aside from milk, the sugar-free mediums were used as checks for the same type of mediums containing sugar. Pellicles, rings, and grease films are produced in these mediums much as in the sugar-containing ones.

*Pellicles, Rings, and Grease Films.*—A few characteristic cultural developments in liquid mediums need consideration at this point. A gray, dry pellicle containing air is formed within 2 days in the case of the *Mycoderma* species. This pellicle is characteristic and is easily distinguished from the type to be described next. It resembles the pellicle produced by *Bacillus subtilis* in bouillon culture. With some yeasts a gradual accumulation of cells on the surface forms a thick, white layer which is moist and easily dislodged. This is usually developed only after several days of growth, and if the tubes are disturbed at intervals it does not appear. In the case of *Oidium albicans* and the sprue organism this type of growth is present and very characteristic.

With certain species a growth of cells takes place at the surface of the medium where it is in contact with the glass. This soon results in a distinct white ring which frequently becomes dislodged when the tube or flask is disturbed and sinks to the bottom where it still retains its original form. This ring is most distinctive at the end of 2 or 3 days, since species which do not form a distinct ring often give this appearance in older cultures due to the accumulation of cells as the liquid evaporates.

By a grease film is meant a 'scum' which is thin and transparent. It resembles a fine covering of oil in the surface and may be seen at the end of a day or 2 only by holding the flask or tube in such a way as to allow the light to reflect from the surface. Tilting the flask serves to render this more distinct since the film remains attached to the glass and shows a distinct grayish layer above the medium. In dextrin yeast water this scum approaches a pellicle in consistency and can be clearly seen at the end of 3 or 4 days.

Turbidity and sedimentation are not distinctive characters in the yeast cultures, although some yeasts form a clear solution after a week of growth as contrasted with the rather turbid condition in other cultures.

*Solid Mediums.*—Streak cultures on agar, carrots, and gelatin are useful mainly in distinguishing yeasts of different colors or shades of color, and such gross types of growth are those mentioned earlier. They are of limited value, however, for the differentiation of species on account of the sudden and inexplicable variations which they undergo. All the pink yeasts studied retained characteristic growths on agar and carrot slants under all conditions.

*Giant Colonies.*—The employment of giant colonies for specific differentiation has been emphasized by Lindner ('05), Will, Geiger, and others. The chief advantage of giant colonies lies in the ease with which they may be photographed and thus serve as comparative charts. The method of developing

the giant colonies is simple. A 200 c.c. Erlenmeyer flask is filled to a depth of 1.5 cm. with a 12% beerwort gelatin. A drop from a fresh beerwort culture of the organism is placed in the center of the gelatin. The colony is allowed to develop for 3 weeks or longer and is then photographed. Geiger ('10) recommends the employment of potato and sauerkraut gelatin in addition to the beerwort gelatin. The types of giant colonies produced are fairly constant according to the authors mentioned. In the present investigation a slightly different method was employed. In addition to beerwort gelatin, glucose agar (2% agar with 2% glucose, and an acidity of +1) was used and Petri dishes of a uniform depth and diameter were substituted for the flasks. Exactly the same amount of medium was placed in each dish, and cultural conditions were kept uniform.

#### SPECIAL STUDY OF 20 REPRESENTATIVE YEASTS

The large number of cultures obtained rendered an intensive study of the entire number impracticable. Moreover, the primary object of the investigation was to work out a scheme by which these yeast-like organisms could be classified in future investigations rather than to make a detailed study of all organisms isolated from the intestinal tract. In order to test the usefulness of the plan proposed, a more intensive study of 20 cultures was made. These were all of the 'white' type except *Saccharomyces hominis* which gives a slimy yellowish growth on agar slants. Most of these were also of the white glistening type since it was desired to undertake the separation of species which resembled each other closely in gross characters. Several named species were also included in this study to serve as 'controls'. An outline of the procedure used is given. Tables 3, 4, 5, and 6 are not arranged in the order of this outline, but are given in the form which was found most convenient in summarizing the results of all the experiments.

#### OUTLINE OF PROCEDURE

1. Morphology
  1. In young cultures of solid and liquid mediums
  2. In old cultures of solid and liquid mediums
  3. In hanging drop cultures
  4. Ascospore formation
2. Cultural Characters
  1. Gelatin-stab cultures
  2. On agar and carrot slants
  3. In liquid mediums aside from milk
    - (a) pellicle, ring, and grease-film formation
  4. Giant colonies on beerwort gelatin and glucose agar
3. Physiology (biochemical properties)
  1. Carbohydrate reactions, fermentation and acid reactions
  2. Litmus milk reactions
  3. Gelatin (included in cultural studies)

In Table 3 there is given a summary of the chief cultural and physiologic characters of the organisms together with the sources of the cultures. The numbers and letters to the left are used in subsequent tables and need some explanation. Culture 2.5 was isolated from a patient diagnosed as sprue and has been treated in some detail earlier in this article. The culture was sent to Dr. Ashford\* who stated that it was the same as his sprue organism. Culture D is a typical sprue organism received from Dr. Ashford, and is included for comparison. The next 14 cultures were isolated from normal persons with the exception of 158, which was from the case discussed previously in which repeated isolations were made from a woman of advanced age who

\* Letter dated Oct. 18, 1916.



TABLE 3  
IMPORTANT CULTURAL AND PHYSIOLOGIC CHARACTERISTICS OF 20 SPECIES SUMMARIZED \*

Number	Source	Type	Gelatin	Milk	Glucose	Sucrose	Maltose	Levulose	Galactose	Lactose
2.5	Sprue	W. g.	V. sc.	Alk.	+	—	+	+	—	—
D	Ashford Sprue	W. g. d.	V. sc.	Alk.	+	—	+	+	—	—
11.5	Feces	W. s.	F.	0	—	—	—	—	—	—
9.1	Feces	W. s.	F.	0	—	—	—	—	—	—
128	Feces	W. i.	V.	0	—	—	—	—	—	—
138	Feces	W. g. d.	V.	Alk.	+	+	+	+	+	—
141.1	Feces	W. d.	V.	0	+	+	+	+	+	—
152	Feces	W. g. d.	V.	Alk.	+	+	+	+	+	—
137	Feces	W. g.	V.	Alk.	+	+	+	+	+	—
141.2	Feces	W. g.	V.	0	—	—	—	—	—	—
215	Feces	W. g.	V. s.	Alk.	—	—	—	—	—	—
138	Feces	W. g.	F.	0	—	—	—	—	—	—
229	Feces	W. g.	V.	Alk. vs.	—	—	—	—	—	—
170	Feces	W. g.	V.	Alk. vs.	+	+	+	+	+	—
172	Feces	W. g.	F.	Alk.	+	+	+	+	+	—
17	Cultural	W. g.	V. sc.	0	+	+	+	+	+	—
M. c.	Cultural	W. g.	V.	Alk.	+	+	+	+	+	—
S. e.	Cultural	W. g.	F.	0	+	+	+	+	+	—
S. h.	Cultural	Y. sl.	L.	Alk.	—	—	—	—	—	—

\* In the table O.a. indicates *Oidium albicans*; M.c., *Monilia candida*; S.e., *Saccharomyces ellipsoideus*; S.h., *Saccharomyces hominis*; W., white; g., glistening; Y., yellowish; d, dull; i, intermediate; Ex., excrecent (a peculiar, dull, lumpy growth); F., filiform; later nodose, L., liquefaction; V., villous; sc., scattered; alk., alkaline; 0, no change; vs., very slight; +, gas; —, no gas; s., slight.



died during the course of the experiment. *Oidium albicans* culture was secured from St. Thomas' Hospital, London, through the kindness of Dr. H. D. Singer of Kankakee. It was isolated from the mouth of a child who was suffering from thrush. The other named cultures were obtained from the "Centralstelle für Pilzkulturen," Amsterdam, Holland. *Saccharomyces hominis* Busse is supposed to be a subculture of Busse's ('95) original organism. Guilliermond states, however, that this organism is supposed to ferment glucose and not liquefy gelatin, neither of which reactions agrees with the results given in Table 3. *Saccharomyces ellipsoideus* is probably a composite species. The data under the head of 'type' give merely the type of growth of young agar slants. Under 'gelatin' is included a rough division into the 3 types outlined earlier. The reactions with sugars are based, in each case, on a number of trials. On the basis of the fermentation of sugars alone, the 20 species may be separated into the following groups:

Group 1. Those not fermenting sugars ....	11.5, 9.1, 128, 215, 172, 138, 137, <i>S. hominis</i> , 141.2
Group 2. Those fermenting all except lactose	158, 152, 229 <i>M. candida</i> <i>S. ellipsoideus</i>
Group 3. Those fermenting glucose, maltose and levulose but not sucrose, galactose, or lactose .....	2.5, <i>D. O. albicans</i> , 147
Group 4. Those fermenting glucose and levulose only .....	141.1, 170
Group 5. Those fermenting glucose, sucrose, maltose and levulose, but not galactose or lactose .....	

It has been found, as mentioned, that there is some variation in the reactions toward sugars. This is especially true of the members of the 3rd group; sucrose and galactose are fermented at times by these organisms, and at other times not.

TABLE 4

CHANGE IN ACID REACTION BROUGHT ABOUT BY 20 CULTURES INCUBATED AT 35 C. FOR 6 DAYS

Number	Raffinose	Lactose	Glucose
2.501 .....	-2.5*	-2.5*	0.0*
D. ....	-5.0	-5.0	-4.5
11.5 .....	-1.0	-1.0	2.0
9.1 .....	0.0	1.0	1.0
128 .....	-5.0	-6.0	-0.5
158 .....	-5.0	-6.5	-3.0
141.1 .....	-2.5	-3.5	-4.0
152 .....	-5.5	-4.5	-5.5
147 .....	-3.0	-2.5	-2.0
137 .....	-2.5	0.0	1.0
141.2 .....	?	-0.5	1.0
215 .....	1.0	0.5	0.0
138 .....	-4.5	0.0	-2.5
229 .....	0.0	-5.5	-4.0
170 .....	0.0	0.0	2.0
172 .....	0.0	0.0	-2.0
<i>Oidium albicans</i> .....	-5.5	-4.5	-5.0
<i>Monilia candida</i> .....	-4.5	-5.5	-6.0
<i>Saccharomyces ellipsoi-</i> <i>deus</i> .....	0.0	1.0	3.0
<i>Saccharomyces hominis</i> ...	1.0	1.0	3.0

\* The results are given in terms of the number of cubic centimeters of  $n/10$  NaOH required to neutralize 100 c.c. of the culture solution, assuming the original reaction to be exactly neutral. If there was a loss of acidity it is expressed by the negative sign.

## PRODUCTION OF ACIDITY AND ALKALINITY IN LIQUID MEDIUMS

Table 4 gives the results of the titrations of cultures in several carbohydrate mediums. These results represent the total acidity or alkalinity at the end of the period indicated in the table. The tests, as here recorded, are intended mainly to indicate whether the organism concerned renders the medium decidedly alkaline or acid after a period of growth.

It is unfortunate that those who have used the reactions with carbohydrate mediums for specific separation in the yeasts have not used more care in recording their results. Usually the descriptions of the methods employed are entirely lacking and the results are recorded in a table as positive or negative, without indicating the length of time after inoculation when the tests were made or the total amount of acid produced. The basic substance employed in making the carbohydrate mediums and the initial acidity are not given in most cases.

Will and Geiger have been more careful in this respect and their results show that the time at which the titrations are made is very important. It is strange that Castellani should find acid developed in most of his carbohydrate mediums with a large number of his *Monilia* species while a decided alkaline reaction in milk is recorded for these same species.

In order to make a more careful study of the changes in acidity during the first month of growth in liquid mediums, 6 of the 20 organisms selected were grown in flasks of yeast water alone and also with the carbohydrates added. These were tested at 3 periods, as indicated in Table 5.

The original acidity of the yeast water was near +1 to phenolphthalein. Uninoculated flasks were always incubated with the others and titrated at the ends of the periods as in the case of the inoculated ones. This precaution was used in order to show that the little evaporation which occurred did not have a decided effect on the results, and to give a large number of check titrations of the original mediums. The titrations were made against  $n/20$  NaOH using phenolphthalein as an indicator. The results are expressed in the number of cubic centimeters of  $n/10$  NaOH it would require to neutralize the acid produced in 100 c.c. of the medium. If the acidity decreased instead of increased it is expressed by a negative sign; in other words, it would take this amount of  $n/10$  HCl to neutralize the alkali in 100 c.c. of the medium. Five c.c. portions were withdrawn by means of sterile pipettes, and the average of 2 titrations was taken in all cases except at the end of the final period, when 3 titrations were made from each flask.

*Discussion of Results.*—The results given in Tables 4, 5, and 6 are evidence that the reactions brought about by the intestinal yeasts are variable, not only among different species but in the same species with different carbohydrates. It is also evident that titrations made at different times during the course of the growth show variations in the same species. The temperature at which the cultures are kept also has an influence on the degree of acidity. That the change in acidity is not due primarily to the carbohydrate present is evidenced by the fact that yeast water without the addition of carbohydrates gave similar results to the yeast water carbohydrate mediums. The addition of certain carbohydrates tend, however, to vary the degree

TABLE 5  
REACTION OF YEASTS ON SUGAR MEDIUMS  
At 35 C.

Name or Number	Yeast Water			Glucose			Lactose
	5 Days	2 Weeks	5 Weeks	5 Days	2 Weeks	5 Weeks	
2.5	-6.0*	-5.5	-5.5	0	-5	-8	-
215.13	-2.5	-2	-4	2	4.5	7	-
170.1	-2.0	-4	-4	2	0.5	1	-
158.3	-6.5	-6	-4	-3.5	-5.5	-5.5	-
152.1	-4.5	-7	-5.5	-2	-5.5	-7	-
<i>Oidium albicans</i>	-8.5	-6.5	-7.5	-3	-5	6	-
Check	0	0	0	0	0	0	-

At 25 C.

2.5	-7.5*	-10	-7	-7	-7	-8	-
215.13	-3.5	-5.5	-7	1	2	0.5	-
170.1	0	-4	-6	2	0	1	-
158.3	-7	-5.5	-5.5	-2.5	-7.5	-7.5	-
152.1	-6	-8	-7.5	-3	-5.5	-7	-
<i>Oidium albicans</i>	-7	-10	-7	-6.5	-6.5	-8.5	-
Check	0	0	0	0	0	0	-

\* The results are given in terms of the number of cubic centimeters of  $n/10$  NaOH required to neutralize of the culture solution, assuming the original reaction to be exactly neutral. If there was a loss of acid expressed by the negative sign before the number. The check had an initial acidity of +0.95, but is recorded order to compare with the other cultures.

of acidity, as shown, for example, by the extreme alkalinity of dextrin solutions.

In general, when yeast water with an initial acidity of +1 is used with or without carbohydrates there is a decided decrease with some species, while others show no marked change in the final reaction in acidity. In spite of the wide variations in results due to these various conditions, there are certain constant differences between species which are useful in specific characterization. It will be seen from Tables 5 and 6 that *Oidium albicans* is a species which decreases the acidity of the mediums in every case. It is also evident that the reactions of Culture 2.5 are of the same general character as those of *Oidium albicans*. On the other hand, Cultures 170 and 215.13 do not bring about a decided decrease in acidity in any of the mediums. These results are constant throughout the entire range of mediums and under both conditions of temperature.

The necessity of stating the period of time the culture has been growing before the test of acidity is made is emphasized by the results obtained after 5 days with Culture 2.5 and *Oidium albicans*.

TABLE 5—*Continued*  
REACTION OF YEASTS ON SUGAR MEDIUMS  
At 35 C.

Lactose		Sucrose			Dextrin		
Weeks	5 Weeks	5 Days	2 Weeks	5 Weeks	5 Days	2 Weeks	5 Weeks
—6	—5	—2.5	—3.5	—6	—7	—7.5	—7.5
5.5	1	—1.5	1.5	—1.5	—3.5	—2.5	—1.5
0.5	0.5	—1	—3.5	1.5	—4.5	4	—1.5
—3	—4.5	—4.5	—4.5	—4.5	—11.5	—9	—10
—3	—4	—6	—6	—6.5	—10	—9	—10
—4.5	—3.5	—0.5	—3.0	—7	—11	—8.5	—8.5
0	0	0	0	0	0	0	0

At 25 C.

—6.5	—6.5	—8	—6	—6.5	—9.5	—11.5	—8.5
—4	—2	—3	1	—5.5	—5	—7	—7
—3	—3	—5	—6	—5	—5	—5.5	—5
—5.5	—5.5	—5.5	—6.5	—7.5	—11.5	—10.5	—11.5
—6.5	—6	—6.5	—6.5	—9.5	—8.5	—11	—11
—9.5	—7	—7.5	—5.5	—4.5	—11.5	—7	—9.5
0	0	0	—0	0	0	0	0

In sucrose and glucose at 35° C. the reactions of these are not different from those of Cultures 170 and 215.13. If the cultures are allowed to grow a month, however, the results are different.

Dextrin yeast water appears to be an unusually good medium for differentiating between those species which decrease acidity and those which produce little change. The differences are very sharp and take place within a short time, especially when the cultures are kept at 35 C.

On the whole, the exact determination of changes in acidity during a period of growth is believed to be of little value on account of the wide variation within a single species even under apparently the same cultural conditions. Increase or decrease in acidity, as revealed by titrations after a week or more of growth, is of value in differentiating species, in that some species tend to constantly decrease the acidity while others bring about no decided change. The initial acidity of the mediums should be determined and stated since this has an important influence on the final reaction. The substances employed in the preparation of the carbohydrate mediums should be stated in all cases since the carbohydrates themselves are not usually the cause of the change in acidity although they may influence the reaction.

TABLE 6  
REACTION IN LITMUS MILK OF 96 YEASTS, 20 DAYS \*

Name or Number	Reaction	Name or Number	Reaction
62	Alk.	231.11	Alk.
27	Alk.	2.501	Alk.
2.6	N.	144.12†	Alk.
161.11	N.	11.131	Alk.
141.12	N.	156.11†	N.
172.11	N.	11.301	Alk.
32a	N.	2.502	Alk.
2.21	N.	215.11	Alk., sl.
128.11	N.	166.11†	Alk.
138.11	Alk.	133.12†	Alk., sl.
11.501	N.	154.11†	Alk.
137.11	N.	15.11	Alk.
251.11	Alk., sl.	10.41	Alk., sl.
170.11	N.	215.12	Alk., sl.
1.13	N.	191.11	N.
118.11	N.	41	N.
129.11	N.	42	Dc., sl.
146.11	A., sl.	147.12	Alk.
9.1	N.	215.13	N. (sl. alk.)
167.11	N.	B	N.
11.401	N.	5.101	N.
139.21	N.	32.11	N.
141.11	N.	61	N.
192.11	N.	163.11†	Alk.
7.11	N.	146.12†	Alk.
2.11	N.	51†	Alk.
209.11†	Alk.	158.11	Alk.
105.11	Alk.	O. albicans	Alk.
119.11†	Alk.	S. path. F.	Alk.
60	Alk.	S. path. C.	Alk.
11.701	Alk.	S. path. B.	Alk.
166.12	Alk.	T. monosa	N.
138.11	Alk.	T. humicola	A.
139.31	Alk.	S. hominis	Alk., sl.
229.11	Alk.	Myc. lactis	N.
152.11	Alk.	Monilia a.	N.
158.31	Alk.	Myc. 78	N.
138.12	Alk.	S. ellips.	N.
1.102	Alk.	S. glutinis†	Alk.
234.11	Alk.	Torula 35	N.
147.13	Alk.	T. rubra†	Alk.
D	Alk.	S. cerevisiae	N.
139.11	Alk.	S. pastorianus	N.
59	Alk.	Monilia?	Alk.
120.12	Alk.	S. albus	Alk.
11.901	Alk.	S. anom.	Alk.
141.21	Alk.	Monilia Simonii	Alk.
11.111	Alk.	M. candida	Alk.

\* In the table Alk. indicates alkaline; N., neutral; no change; A., acid; sl., slight degree of change; Dc., decoloration; O., Oidium; S., Saccharomyces; F., Foulerton; C., Curtis; B., Binot; T., Torula; Myc., Mycoderma; Monilia Simonii, used for convenience only, and does not signify that this species is named from a botanic standpoint.

† Pink yeasts.

#### COMPARISON OF YEASTS ISOLATED FROM THE DIGESTIVE TRACT WITH DETERMINED PATHOGENIC AND NONPATHOGENIC SPECIES

The question naturally arises whether the yeasts isolated from the digestive tract are similar to, or identical with, those associated with disease. The present investigation did not include extensive studies of pathogenic species, but a number of these were secured and used for comparison, not only with the intestinal yeasts, but also with those secured from other sources. Saccharo-



myces hominis, *Torula humicola*, *Blastomyces dermatitidis*, *Oidium albicans*, *Torula rubra*, *Monilia* X of Ashford, Simon's ('16), Binot's ('03), Curtis ('95), and Foulerton's ('99) pathogenic yeasts were those most carefully studied. The nonpathogenic determined yeasts used are given in Table 2.

The pathogenic yeasts most nearly resembling the intestinal fungi isolated were *Oidium albicans*,\* *Monilia* X of Ashford, and Simon's species. Ashford's *Monilia* has many characters in common with *Oidium albicans*, and considering the wide variation in cultural and biochemical characters which this species apparently undergoes it is questionable whether the fungus associated with sprue is a distinct species. Ashford ('15c) writes that his organism is not *Monilia albicans*, but does not state the grounds on which he bases his assertion. Culture 2.5, isolated from a case diagnosed as sprue, is the same as Ashford's *Monilia* X. This was the only culture in the entire number isolated which closely approached Ashford's sprue organism, with the exception of Culture 147.103 which gave the same reactions in sugar mediums but differed in its morphologic characters and type of giant colony formed. In general, however, the type of growth produced by the sprue organism resembles a large number of the yeasts studied in that it forms a white, glistening, firm surface which remains moist, heaped, and chalk-white.

The pathogenic yeast of Simon also has many characters in common with the sprue and thrush organisms. It forms the same type of surface growth and produces similar reactions in sugar mediums, as well as the peculiar villous growth in gelatin-stab culture. It was received too late in this investigation to make a complete study of all its biochemical characters.

*Blastomyces dermatitidis* is decidedly different in most of its characters from any of the yeasts isolated, as well as from the other pathogenic yeasts. The production of a dry, fluffy, aerial mycelium, commonly formed in old cultures of this organism, is a character not possessed by any of the yeast-like organisms under consideration. There is also a marked tendency of this fungus to grow beneath the surface of the agar slant forming a tough, leathery, mycelial layer from which arise tufts of hyphae, strongly resembling Indian wigwams. The budding condition is not predominant in this fungus and, as stated later, it is not to be considered as a yeast.

The remaining pathogenic yeasts mentioned above differ from those isolated from the intestinal tract in their cultural characters especially. While the majority of the intestinal species form a distinctly chalk-white growth on agar slants which becomes only slightly darker with age, *Saccharomyces hominis*, *Torula humicola*, and the pathogenic yeasts of Curtis, Foulerton, and Binot develop more or less slimy growths which become brown, yellowish-brown, or ash-gray in color. There are also marked morphologic distinctions between these species and those of the intestinal tract.

A comparison of the nonpathogenic yeasts listed in Table 2 with those from the intestinal tract demonstrates that these 2 groups have many characters in common. The chalk-white streak on agar slants is commonly developed by a number of species in both groups, while the spreading *Mycoderma* type of growth occurs in a number of the intestinal species, as well as in the true

\* The specimen of *Oidium albicans* used in this study was obtained from the St. Thomas Hospital in London and was stated to be a typical culture from the tongue of a child suffering from an undoubted case of thrush. (Letter from Dr. Frank Babtree, dated March 3, 1917.) This form does not liquefy gelatin readily, nor does it clot milk. Castellani ('16) describes 7 'forms' of this species none of which liquefy gelatin, while 6 of them clot milk. He states that the name '*Monilia albicans*' should be reserved for the form which clots milk and liquefies gelatin. He regards these forms as being different species, although he does not name them, and is of the opinion that there is a plurality of species causing thrush.

*Mycoderma* species studied. Many of the red yeasts from the intestinal tract were indistinguishable from *Saccharomyces glutinis* and *Torula rubra* in gross characters. However, it was not possible to demonstrate that any of the species isolated were identical with *Saccharomyces cerevisiae*, *S. ellipsoideus*, and other culture yeasts. Several of the isolated species, for example, 152 and 158.3, were very similar in their biochemical properties to *Monilia candida*.

A number of wild yeasts were also isolated from the air and from decaying fruits and vegetables. These were even more closely related to the intestinal organisms than were the nonpathogenic yeasts listed in the table. In the light of the feeding experiments described later, it is certain that these yeasts, when ingested with the food, will pass through the alimentary tract in a living condition and may be isolated from the feces. It is probable, therefore, that the majority of the yeasts isolated in this investigation are these wild species so common in nature. That there is no one yeast species common to the intestinal tract of man is demonstrated by the fact that the majority of the numerous cultures isolated showed, from even a superficial study, that they were of distinct species. It is interesting to know, in this connection, that *Saccharomyces guttulatus* is constantly found in the intestinal tract of rabbits. This species was not met with in the course of the present investigation, and is certainly not common in the intestinal tract of man.

#### FEEDING EXPERIMENTS WITH YEASTS

The most exhaustive feeding experiments with yeasts were those carried out by Neumayer ('91) referred to in a previous section. In these experiments, however, the investigator used only a few species of yeasts and these were, for the most part, the usual fermenting forms of commerce. Neumayer proved that the various secretions encountered throughout the alimentary tract were either favorable for the development of the yeasts or neutral in their influence. Those secretions which were harmful had little influence on account of the fact that the yeast cells quickly passed beyond their influence, and the short period during which the cells were in contact with them was not sufficient for harmful action. He also observed that the yeasts found in the feces were not in a budding condition and that many of them were dead. He proved that, while the sum of the conditions in the digestive tract was not favorable for development and multiplication of these fungi, they still were able to survive in comparatively large numbers. The result of his experiments shows that the ordinary fermenting yeasts and wild yeasts found in beer and grape must are not harmful when fed alone, but may cause serious illness when fed with fermentable carbohydrates.

Few other investigators have attempted to prove the pathogenicity of yeasts by feeding, seemingly preferring to inject the organism into the blood stream or into the peritoneal cavity. Ashford ('16) has made a complete series of experiments on laboratory animals with his sprue organism. In his feeding experiments he found that the fungus is

pathogenic only when recently isolated or after it has passed through some susceptible animal. This may explain the negative results of others in their injection experiments.

In order to test and extend the results obtained by Neumayer and Ashford, and especially to gain more insight into the fate of the ingested yeasts, a number of experiments were undertaken with the yeasts previously isolated or with certain commercial yeasts having well defined characters.

The yeasts selected were those having some distinctive character so that they could be quickly recognized when reisolated from the feces. The following species were used:

1. *S. glutinis* (red)
2. 51 (red and forming a peculiar wrinkled colony)
3. *S. anomalus* (distinctive ascospores)
4. 11.5 (produces ascospores of a peculiar type and has a distinctive growth)
5. D (a culture of Dr. Ashford's sprue organism)

A number of other species were used but since the results do not differ in any way from those here obtained they are not included. The methods used and the results obtained for the first 4 organisms given were identical and will be discussed together. The yeasts were grown on an agar slant for several days and then a mass about the size of a grain of corn was swallowed. Cultures were always ingested within a few hours after the passage of stools and in most cases the first sample of feces was obtained within 30 hours. Samples also were taken from stools just previous to feeding and plates were made from these in order to exclude the possibility of any species being present in sufficient quantity to interfere with the experiment. Since the person used had been previously tested a number of times for other isolations, it was known that few yeasts were normally present in the feces.

Ten plates were made from 10 different portions of the stools in the case of each sample. Subsequent stools during the course of a week or 10 days were tested in the same manner. After feeding 1 species, a period of several days was always allowed before the next feeding, in this way eliminating the possibility of confusing the 2 species fed. The tests were made on a person in good health and showing a history free from gastro-intestinal troubles in recent years. No effort was made to control the diet which consisted of the normal mixed food of a healthy person with a large amount of carbohydrates.

The first stools following the feeding always contained large numbers of the particular yeast ingested. All the plates gave numerous colonies and most of them usually showed over 50% positive results, while many developed colonies of the yeast ingested at 100% of the points of contact. The yeasts seemed to be fairly evenly distributed throughout the stools, although microscopic examination showed them more abundant in some mounts than others. Under the microscope the majority of the cells were seen to be of a faint yellow color and few of them were budding. They did not appear to be in an active growing condition, but most of them seemed to be living. A careful examination of the plates was always made to discover, if possible, foreign types of yeasts. In several cases when pink yeasts were fed, 3 or 4 white colonies in the entire 10 plates appeared, and the reverse was true when white yeasts were ingested.

This result was to be expected in the light of the previous experiments on this person.

In 2 of the first 4 experiments the plates taken from the 2nd stools, that is, about 24 hours later, showed a few scattered colonies of the same species that was ingested. In subsequent stools no trace of the original yeast could be found.

The fact that the yeasts were found distributed fairly evenly throughout the stools is to be explained only on the ground that the ingestion of the organisms so soon after the passage of the previous stools permitted a mixture of the cells with the food in the stomach. It is probable that the yeasts are able to multiply in the stomach, due to the favorable acid condition and the lack of harmful secretions. They are then passed on with the food and from this point probably do not find conditions conducive to rapid multiplication. The absence of these yeasts in subsequent stools indicates that they pass along with the food, and that the few which do remain in the stomach or intestine are not able to multiply to any great extent before they are finally passed out in subsequent stools. The fact that they are not able to establish a foothold anywhere along the way is of fundamental importance, since the rich carbohydrate food which passes through the digestive tract would give them an opportunity to produce harmful products within the body.

The person to whom these various species were fed suffered no inconvenience during the course of the experiments. When yeasts with power of active fermentation were fed, as for example, *Saccharomyces ellipsoideus*, there sometimes resulted a slight 'full' feeling a few hours later, but this was not of long duration, and apparently no large amount of gas was developed either in the stomach or the intestine.

A more detailed account of the feeding experiment with the sprue organism from Dr. Ashford is necessary. This culture was pronounced a typical sprue organism by Dr. Ashford and agreed fully with his description of the typical sprue yeast. On the basis of his results with animals, he expresses the opinion that the virulence of the yeast is lost after being on mediums for some time, and this virulence can be regained only by passing the culture through some susceptible animal. Since my culture had been on mediums for some months and had presumably, to a large degree lost its virulence, it was decided to test its period of life in the digestive tract of the person previously fed with the other culture yeasts. Therefore, a mass of about 1 gm. was taken from the surface of an agar slant and ingested without other food. This feeding took place a few minutes after the passage of stools which were saved and tested, with negative results. No pain was felt during the first day after feeding and no intestinal disturbance was noticed. Unfortunately, there was no passage of stools for over 48 hours, and some of the symptoms subsequently described may have been due to the constipated condition of the person. The first stools secured were very hard and lumpy. The results of plating were the same as in other feeding experiments, except that the first inch of the feces gave entirely negative results.

After the first 48 hours considerable discomfort to the person resulted. An 'uneasy' feeling in the intestine, with pain in the region of the stomach, and 'heartburn' were noticed. However, at no time was there excessive gas in the stomach or intestine, and no tendency toward a diarrheal condition. The next passage of stools was also delayed, occurring 36 hours after the first. This was plated and gave a large percentage of the type ingested. There were several colonies of pink yeasts in the plates from the 1st stools but none in those of the 2nd. *Penicillium* colonies in considerable numbers appeared in these plates. All parts of the 2nd sample gave the sprue organism and the



colonies were evenly distributed. The 3rd passage, 24 hours later, was of normal consistency, although the pains in the region of the intestine were still acute. Samples from this stool were plated but the agar was unsatisfactory and no accurate results were obtained. Sufficient colonies appeared, however, on 1 good plate to indicate that the organisms were still present. The 4th stool gave a large percentage of the sprue organism. The number of positive contacts was somewhat reduced in these plates, but several of them yielded over 50%. This 4th sample was taken 6 days after the ingestion of the yeasts and is the only case in which more than 2 successive stools gave colonies of the organism ingested. The next 2 stools were not saved on account of the pressure of other work, but the pain in the stomach and intestine disappeared and subsequent stools gave entirely negative results. The rather sudden disappearance of the yeasts was unexpected and difficult to account for.

During the course of this experiment the person fed on his normal diet. Carbohydrates did not constitute as large a percentage of the food as normally, but were not much less than the average person would use.

The most important conclusion to be drawn from this experiment is that this yeast differs from all others investigated in that it was able to remain and seemingly to develop in the alimentary tract. It is not conceivable that the small number of yeasts fed could continue to give positive results over this long period unless they did multiply during the time they were in the intestinal tract. Unfortunately, no attempt was made to determine whether or not they were present in the stomach after the first 48 hours. While the other yeasts investigated seemed to pass through the alimentary tract as so much waste food, except for a possible brief period of multiplication in the stomach, this organism undoubtedly was able to carry on its normal processes and secure at least a temporary foothold somewhere in the digestive canal. The mere presence of active yeasts in the intestinal tract for a long period of time would, undoubtedly, give rise to harmful by-products, as maintained by Neumayer. If, in addition to this, they are able to establish themselves in the mucous membrane, as do the thrush and sprue organism, their presence would lead to serious results.

#### THE CLASSIFICATION OF BUDDING FUNGI

The fungi having budding stages in their life cycles are of 3 types: (1) those in which budding is a secondary phase, usually occurring under unusual or abnormal conditions, or during only a short period of the life cycle of the organism; the primary phase consists of a distinct mycelial development; (2) those in which budding is the primary phase, occurring under all the usual conditions of growth, but always with mycelial formation, more or less rudimentary, as a secondary phase; (3) those in which budding is the only known method of vegetative multiplication.



The first group includes a number of species from diverse orders of fungi. The smuts (*Ustilaginales*) commonly produce large numbers of cells from the primary sporidia by the budding process. The basidiospores of species of *Calocera*, *Tremella*, and many other basidiomycetes, and the ascospores of certain ascomycetes, such as *Sphaerulina intermixa*, and various species of *Taphrina*, produce new cells by budding. Hesler ('16) describes the production of 'microconidia' by budding of the hyphae in *Physalospora Cydoniae*, and Alwood ('98) records the occurrence of a yeast-like form in cultures of this same species, but does not account for its origin. *Mucor racemosus* when placed in sugar solutions forms budding cells similar to yeasts in a budding condition. *Dematium pullulans* produces an extensive mycelium from the filaments of which there are numerous buds. In some fungi the budding stage is more prominent than in those cited; for example, the organism described by Gilchrist and Stokes ('95), Ricketts ('01), and others as causing a serious skin disease, and commonly called *Blastomyces dermatitidis*, has an extensive budding phase in its life cycle. Under certain, little understood conditions it continues budding for a long period. Sooner or later, however, it forms a white, fluffy, aerial growth which sharply distinguishes it from the yeast-like fungi.

To the 2nd group belong those forms which are predominantly budding. They form a firm, moist growth on solid mediums and rarely produce aerial mycelium. Under certain conditions they form elongated cells which become septate but rarely branch. *Monilia candida* and species of *Endomyces* are examples of this group.

The last group, which includes the strictly budding species, embraces the greater part of the *Saccharomycetaceae*, or the spore forming yeasts, and those commonly placed in the genus *Torula* by writers following Hansen's definition of this genus. This and the second group are subdivided on the basis of whether or not ascospores are known to occur in their life cycles.

#### TAXONOMIC DISCUSSION

The position of the asporogenic yeast-like organisms among Fungi has not been satisfactorily determined. As will be shown, they have been placed in a number of diverse genera under the different schemes of classification.

The systems of classification of the fungi most generally followed by mycologists and botanists are those presented by Lindau, in Engler and Prantl's "Die Natürlichen Pflanzenfamilien," and by Saccardo in his "Sylloge Fungorum." Since the classification of Fungi imperfecti is practically the same in the 2, this system will be spoken of as Saccardo's classification. In this system there are 4 classes of fungi: *Phycomycetes*, *Ascomycetes*, *Basidiomycetes* and *Fungi imperfecti*, the last class being a provisional one for the inclusion of all the fungi not having a perfect or sexual stage, or this stage not having been discovered as yet. The endospore forming yeasts are placed in the *Ascomycetes* under the family *Saccharomycetaceae*, while those not forming spores should logically be placed in the *Fungi imperfecti*. The *Fungi imperfecti* are divided into 3 orders, *Sphaeropsidales* (*Phoma-*

tales), Melanconiales, and Moniliales (Hyphomyceteae). The 3rd order includes 4 families, Moniliaceae (Mucedineae), Dematiaceae, Stilbaceae, and Tuberculariaceae. We are concerned with the first of these families only since the yeast-like fungi under discussion would be included in this group on account of their color and simple organization.

Frank ('86) has separated the fungi into the following orders: Ascomycetes, Basidiomycetes, Zygomycetes, Phycomycetes, and Blastomycetes. He also has a provisional group of the Fungi imperfecti, but includes all the budding fungi, whether spore forming or not, in the order Blastomycetes.

Vuillemin ('10) has suggested another classification for the mycoses of animals. He has 2 orders, Hyphales and Siphales, the latter including all phycomycetes of the Saccardian classification. The Hyphales are divided into Microsiphones, Conidiospores and Thallospores. Conidiospores are those fungi which form their conidia as distinct fructifications on more or less specialized branches of the mycelium (thallus), while Thallospores are those which form their reproductive bodies directly from the thallus or mycelium. The latter group he divides into Arthromycetes and Blastomycetes, according to whether the spores are produced as a part of the body of the thallus or by budding of the cells. The fungi under discussion, therefore, would be placed in Blastomycetes. It is from the use of these various systems by different writers that so much confusion has arisen in the classification of the yeast-like organisms.

The last 2 classifications mentioned place considerable emphasis on the building process, although in Frank's system this is not the only distinguishing character of the group. Vuillemin, working with animal pathogens, naturally regarded this as a very important character.

I believe that the importance of the budding process has not been sufficiently emphasized in the classification of Saccardo. None of the authors mentioned has laid sufficient stress on the essentially unicellular condition in this group.

Since the system of classification of Saccardo is so generally established, it is thought best to adapt it to the forms under discussion. It is believed that the family Moniliaceae should include a subfamily into which those forms which are essentially unicellular and budding can be placed and that the description of the family should be emended to include such subfamily. For the present, however, they will be placed in the tribe Oosporeae, as defined by Lindau.

The question of the generic position of the asporogenic yeasts is still more confusing and a special discussion of this point will be entered into here.

The yeast-like fungi without endospore formation have been placed by various writers in one or another of the genera listed below. Those genera are excluded which include endospore formation as an essential feature of their classification. Genera such as *Saccharomyces* and *Endomyces* have been used to include the asporogenic forms, by a number of authors, on the same grounds that certain rusts are included among Basidiomycetes, although their 'perfect' forms are not known. Each genus in the accompanying list is followed by the name or names of the writers who have used it most prominently to include the typical asporogenic yeasts.

*Blastomyces* (Gilchrist and Stokes)  
*Cryptococcus* (Guilliermond, Vuillemin)  
*Hormiscium* (Bonorden)  
*Monilia* (Castellani, Ashford, etc.)  
*Mycoderma* (Hansen, Will)  
*Oidium* (Robin, Cao)  
*Pseudomonilia* (Geiger)  
*Pseudosaccharomyces* (Will)  
*Torula* (Hansen, Will)

The classification used by de Beurman and Gougerot ('09) includes the genera *Zymonema*, *Atelosaccharomyces* and *Parasaccharomyces*, all of yeast-like character. These will be discussed further.

Accepting as final the rulings of the Brussels Congress on the naming of the fungi, the writer has not attempted to antedate Fries ('21-'32) in the discussion of these genera, except where earlier descriptions aid in clearing up certain vague points in the descriptions of later writers.

The genus *Torula* has been used by practically all investigators, interested especially in fermentative industries, to include all the asporogenic yeasts which do not form a distinct pellicle in liquid mediums, those forming a pellicle being placed in the genus *Mycoderma*. Hansen, Will, Guilliermond, and Lindner accept this interpretation of the genus. On the other hand, the mycologist regards those dark colored forms which form chains of spores resembling a necklace of beads as true *Torula* species. Thus Saccardo and Lindau have both placed this genus in the family Dematiaceae which is made up of dark spored forms. The genus *Torula* was founded by Persoon and emended by Fries, who used as his type species *Alternaria tenuis* of

Persoon. This is described by Persoon ('22-'28) as having dark-colored spores, which character justifies later authors in placing this genus in the Dematiaceae. Turpin ('38) studied the budding yeast plants in beer and named them *Torula cerevisiae*. He had the impression that they were a stage in the life of a plant which, in the presence of abundant oxygen gave first *Mycoderma cerevisiae* and later *Penicillium glaucum*. Hansen and others have used Turpin's description as a basis for including all asporogenic yeasts of this character in *Torula*.

Sumstine ('13) and Vuillemin ('11) discuss at length the genus *Monilia* from a taxonomic standpoint. The former states that, judging from Persoon's description and synonymy, *Monilia* is congeneric with *Aspergillus* and *Penicillium*. Vuillemin also holds that most of the species originally included by Persoon under the genus *Monilia* have been shown to belong to *Aspergillus*, *Penicillium*, or *Torula*. Fries takes as the type of the genus, *Mucor caespitosa* Linn., which is the same as *Monilia digitata*. This, from his description and the statements of Persoon, is an *Aspergillus*, as the name itself would suggest. Thus there is no doubt but that Fries had an *Aspergillus*-like fungus in mind as the type of the genus *Monilia*.

Vuillemin, however, taking *Monilia fructigena* as the type of the genus tries to show that this is very similar to *Monilia candida* (*M. Bonordenii* Vuil.) in its process of spore formation, and in turn he accept Plaut's ('87) conclusion that *Monilia candida* is the same as the thrush organism, *Monilia* (*Oidium*) *albicans*. He bases his conclusions on the fact that the spores of *Monilia fructigena* are blastospores rather than conidiospores. Thus he attempts to justify placing all the yeast-like fungi of the *Oidium albicans* type in the genus *Monilia*. But anyone who has made a study of *Monilia* (*Sclerotinia*) *cinerea* and *fructigena* is familiar with the fact that they produce a distinct aerial mycelium, and that the conidia are of fairly uniform size and germinate by means of a true germ tube. All of these characters are lacking in *Oidium albicans*. If Vuillemin's conclusions were in accord with the rules of nomenclature, and we accepted *Monilia fructigena* as the type of the genus, there would be sufficient basis for discarding it for the yeast-like forms under discussion. On the other hand, if the type of this genus is *Aspergillus*-like, then the forms under consideration could not be included.

The genus *Oidium* was founded by Link in 1809. Fries gives as the type of this genus *Mucor leprosus* Linn., and as a synonym of this *Oidium aureum* Link, thus accepting Link's type species. This fungus



is well known and has a distinct septate, aerial mycelium, and sporophores, characters which serve to eliminate it from the present discussion.

The genus *Blastomyces* has led to great confusion in that the budding fungi are often known as blastomycetes. Though Frank created an order which he called 'Blastomycetes,' he did not have a genus of that name in the order. Costantin and Rolland ('88) created a new genus, *Blastomyces*, for a fungus which is not at all yeast-like in character. Gilchrist and Stokes ('96) named a budding organism isolated from a skin disease, *Blastomyces dermatitidis*, and Roncali ('95) also used this generic form for a budding organism isolated from a carcinoma of the ovary. The former organism is yeast-like only during a part of its life history, later forming a distinct aerial mycelium. The latter is very similar to the organisms studied in this investigation. Medical writers in general have been inclined to use this name in a loose sense for all pathogenic yeast-like fungi. The genus is tenable neither for these organisms nor for the type represented by Gilchrist and Stokes' fungus.

The genus *Hormiscium*, erected by Kunze and Schmidt in 1817, and used by Bonorden ('51) to include a number of the fermenting yeast-like organisms, has been emended and transferred to the *Dematiaceae* by Saccardo.

*Cryptococcus* was erected by Kützing ('33) as a genus of the algae. He lists 1 species, *Cryptococcus mollis*, and does not include a specific description. The generic description is as follows: "*Globuli mucosi hyalini non colorati, in stratum indeterminatum muscosum facile secedens sine ordine aggregate.*" In a later publication he places *Saccharomyces cerevisiae* in this genus. Vuillemin ('01) has, unfortunately, selected this name for a genus to include all the pathogenic yeasts. Guilliermond ('12) has accepted Vuillemin's interpretation of this genus, and places in it all yeasts not forming spores which are associated with animal disease. The genus *Cryptococcus* is considered valid for those asporogenic yeasts which do not form a distinct septate mycelium, and will be used in the future discussion for such species.

De Beurmann and Gougerot ('09) have discussed in detail the classification of the mycoses of animals. They call attention to the great confusion which exists, especially among medical writers, regarding the proper botanic position of the various types of budding fungi causing disease. They present a scheme of classification which includes all the described species of budding fungi associated with animal



disease. The accompanying summary of their system includes only a discussion of the genera involved.

1. *Saccharomyces*, Meyen, 1838. In this are included those yeasts which have round, oval, elliptical, or elongated cells, with vegetable reproduction by budding only, and ascospore formation always present.

2. *Atelosaccharomyces*. de Beurmann and Gougerot, 1909 (genre provisoire). This genus, in all respects, except in the formation of ascospores, is exactly like *Saccharomyces*, that is, it is the 'imperfect' genus corresponding to *Saccharomyces*.

3. *Parasaccharomyces*.\* de Beurmann and Gougerot, 1909. (Emend Anderson.) Similar to the preceding genus except that there are elongated cells and rudimentary mycelial threads.

4. *Zymonema*. De Beurmann and Gougerot, 1909. Thallus composed of a mixture of round, oval, or elliptical budding cells, and distinct, branched septate mycelial elements. The hyphae may break up into short rectangular cells as in *Oidium lactis*, or may form either intercalary or terminal chlamydospores.

5. *Endomyces*. Rees, 1870 (Emend). Thallus formed of branched, budding articles. Ascus solitary at the end of the filament and containing 4 hemispheric or reniform, hyaline ascospores.

Of the 5 genera the first and last form ascospores and their characters are well established. The 2nd, 3rd and 4th genera contain the budding forms not producing ascospores, and it is with these that we are especially concerned. *Atelosaccharomyces* as defined by these authors corresponds to the genus *Cryptococcus* of Kützing, and on this account the earlier name, *Cryptococcus*, has been substituted in the following discussion for *Atelosaccharomyces* of De Beurmann and Gougerot. *Parasaccharomyces*, though not well defined by these authors, is evidently intended to include such forms as *Monilia candida*, in which the budding phase predominates, but rudimentary septate hyphae are produced. A number of species of this character were studied in the present investigation, some forming only a few unbranched elongated elements which rarely become septate, others producing a scantily branched but abundant mycelium with septate hyphae (*Monilia candida*). The mycelium is never aerial nor dry in character in these species. It has been thought best to accept the genus *Parasaccharomyces* with this more complete characterization rather than to add to the confusion by erecting another genus.

The genus *Zymonema* was erected by De Beurmann and Gougerot to include such species as '*Blastomyces dermatitidis*' in which the

\* Parasites mal classes, proches des *saccharomyces*. Mêmes caractères que les *atelosaccharomyces*, c'est-à-dire cellules rondes ou ovoïdes ou allongées, bourgeonnants sans ascus connus. Mais les formes de transition avec les parasites suivants (ébauches filamenteuses, formes oidiennes) sont plus marquées et indiquent l'éloignement du genre bien défini des *saccharomyces*.

budding phase is secondary in importance, the final result of growth in culture being a markedly branched, aerial mycelium. They propose the name *Zymonema Gilchristi* for the organism described by Gilchrist and Stokes ('95), and more thoroughly investigated by Ricketts ('01) and others. This name should be adopted for the fungus, in order to do away with the confusion which has arisen in recent years from the use for it of such generic names as *Blastomyces*, *Oidium*, and *Cryptococcus*.

The accompanying key includes the genera of budding fungi, in which the budding phase is predominant. This includes not only the yeasts without spore formation, but also those developing ascospores.

#### KEY TO GENERA OF BUDDING FUNGI

##### I. Ascospores known:

Vegetative cells single or attached in irregular colonies, mycelium not developed, ascospores formed within isolated vegetative cells ..... (Saccharomycetaceae\*)

Spores globose or ovoid:

Spores on germination forming typical yeast cells:

Ascus formation preceded by the conjugation of gametes..... 1. *Zygosaccharomyces*

Ascus formation not preceded by the conjugation of gametes:

Spore membrane single..... 2. *Saccharomyces*

Spore membrane double..... 3. *Saccharomycopsis*

Spores on germination forming a poorly developed promycelium..... 4. *Saccharomycodes*

Spores pileiform or limoniform, costate.... 5. *Willia*

Spores hemispheric, angular or irregular in form, on germination forming an extended promycelium..... 6. *Pichia*

Vegetative cells produced predominantly by budding, but forming a mycelium under some conditions, asci terminal or intercalary, differentiated from the mycelium..... 7. *Endomyces*

##### II. Ascospores not known, i. e., Fungi imperfecti:

Heavy, dry pellicle formed on liquid mediums.. 8. *Mycoderma*

No distinct pellicle formed:

Vegetative cells forming a septate mycelium under exceptional conditions but predominantly budding..... 9. *Parasaccharomyces*

Vegetative cells formed only by budding. Cells apiculate, limoniform..... 10. *Pseudosaccharomyces*

Cells frequently elongated into narrow, nonseptate hyphal threads..... 11. *Pseudomonilia*

Cells typically yeast-like..... 12. *Cryptococcus*

\* The genus *Schizosaccharomyces*, which does not bud, and the relatively unimportant genera, *Monospora* and *Nematospora*, are not included in this key.

## DESCRIPTIONS OF NEW SPECIES OF YEASTS

Over 100 cultures of unknown yeast-like fungi were studied during the present investigation. The cultural and morphologic characters of all these were studied sufficiently to demonstrate that there were few absolute duplications in the entire number of cultures. A detailed investigation of a group of these cultures has been presented in a previous section. Specific descriptions of some of these, together with a few other interesting forms, will be given. Cultures of the described species have been sent to the American Museum of Natural History, New York, the Centralstelle für Pilzenkulturen, Amsterdam, Holland, and the United States Department of Agriculture, Bureau of Plant Industry. An additional set together with dried type material has been deposited in the herbarium of the Department of Botany, University of Illinois.

## PSEUDOSACCHAROMYCES STEVENSI SP. NOV.

*Morphology.*—In both young and old cultures the cells are narrowly elliptical, oblong, or apiculate; cytoplasm, very granular; vacuoles, not distinct except in old, swollen cells; no elongated cells or false mycelium are formed under any condition of culture. Budding occurs only at ends, by elongation and swelling of the apiculate portion. The size is  $2 \times 5$  microns.\* No endospores are formed. (Plate 3, Figs. 10, 11.)

*Cultural Characters.*—On glucose agar the streak is filiform, glistening, white, flat, and smooth. The growth is slow, and the colony becomes dirty-gray with age. In gelatin no liquefaction occurs; the growth is filiform. In beerwort and sugar mediums there is slow development, with no evidence of growth except a slight sediment. The giant colonies are very small. (Plate 6, Fig. 9; Plate 8, Fig. 18.)

*Physiologic Properties.*—There is no fermentation of glucose, levulose, sucrose, lactose, raffinose, galactose, or maltose. No decided change in acidity occurs in these sugars, dextrin, or yeast water. There is no change in litmus milk.

The culture was isolated from human feces. (Culture 9.101; Type Specimen 1; Type Slide 9.)

## ZYGOSACCHAROMYCES BISPORUS SP. NOV.

*Morphology.*—In young liquid cultures the cells are oval or ovate; in old cultures they assume various forms with numerous conjugating, but usually no sporulating cells. Elongated cells are common, but there is no mycelial formation. Budding occurs from end or side. The size is  $4 \times 6.5$  microns. Spore formation occurs on carrot slants at room temperature. Conjugation is most common previous to spore formation, but parthenogenesis is not rare. There are 2-4 ascospores, most commonly 2. (Plate 4, Fig. 8.)

*Cultural Characters.*—On glucose agar the growth is spreading, dull, flat, and white; later it becomes brownish with small, scattered, wart-like prominences and more glistening surface. There is a filiform growth in gelatin stab and liquefaction in beerwort gelatin in 3 weeks. Pellicle is present on beerwort and some sugar mediums. Giant colonies may be seen in Plate 6, Figure 6.

\* The size given is not the average of a large number of measurements, but is the result of an attempt to select a single cell which will represent the size and proportions of the most numerous cells of a fairly uniform character. This plan is believed to give more definite results than could be obtained by measuring numerous cells and averaging these. This conclusion is based on a study of both methods.

*Physiologic Properties.*—It does not ferment glucose, sucrose, levulose, maltose, galactose, or raffinose. No decided change in acidity occurs in these mediums. There is no change in litmus milk.

The culture was isolated from human feces. (Culture 11.501; Type Specimen 3; Type Slide 11.5.)

CRYPTOCOCCUS VERRUCOSUS SP. NOV.

*Morphology.*—In young liquid culture the cells are oblong, narrowly elliptical or oblong-elongated; in old cultures elongated cells are common, with several 'oil' globules in each cell. The size is  $3 \times 9$  microns. Budding occurs from shoulders, ends, or sides. No endospores are formed. (Plate 5, Figs. 1, 2.)

*Cultural Characters.*—On glucose agar slant there is at first an even, filiform, glistening, white, smooth growth; later it becomes dull, brittle, verrucose, and pulvinate. On carrot slant the growth is more profuse, with verrucose character more pronounced, and with chalky-white surface. There is a filiform or nodose growth in gelatin stab, with no liquefaction. On sugar mediums and beerwort, after 2 days, a few small, white patches appear on the surface, later becoming larger, dry, and very firm; at first they remain separate, but later coalesce. Giant colonies may be seen in Plate 6, Figure 10, and in Plate 8, Figure 14.

*Physiologic Properties.*—It does not ferment glucose, levulose, sucrose, maltose, galactose, lactose, or raffinose. No decided change in acidity occurs in these sugars. Litmus milk becomes very slightly alkaline after several weeks.

The culture was isolated from human feces. (Culture 172.1; Type Specimen 2; Type Slide 172.)

The dry brittle character of the colonies on solid mediums, the formation of the isolated, white patches on all liquid mediums, and the peculiar type of cells, clearly distinguishes this yeast from any other studied.

CRYPTOCOCCUS AGGREGATUS SP. NOV.

*Morphology.*—In both young and old cultures the cells are mostly globular or slightly oval. No elongated cells are formed. Budding occurs from any point on the cell, usually several buds arise from each cell; in old cultures buds are commonly formed in large numbers about a single enlarged cell. The size is 3.5 microns. (Plate 4, Figs. 1, 2.)

*Cultural Characters.*—On glucose agar slant the growth is filiform, convex, glistening, smooth, chalk-white, and firm. In old cultures the surface remains smooth, with even edges and no darkening in color. Filiform, later nodose growth occurs in gelatin stab, with no liquefaction. No pellicle or ring is formed in beerwort or liquid sugar mediums. The surface of the giant colonies on glucose agar plates remains remarkably smooth, only dim, concentric lines appearing. (Plate 6, Fig. 12; Plate 8, Fig. 19.)

*Physiologic Properties.*—There is no fermentation in glucose, sucrose, levulose, maltose, galactose, lactose, or raffinose yeast water. No decided change in acidity occurs in these sugar mediums. Litmus milk becomes very slightly alkaline after 3 weeks.

The culture was isolated from human feces (Culture 215.103; Type Specimen 3; Type Slide 215).

Two other cultures, 215.101 and 215.102, isolated from the same person were compared with the foregoing species and found to be identical. The isolations were made from the same sample of feces but from different colonies.



## CRYPTOCOCCUS OVOIDEUS SP. NOV.

*Morphology.*—Cells in young cultures are round or oval, and fairly uniform in size and shape; in old culture cells are oval or broadly elliptical, varying markedly in size and with few budding cells. There are no elongated cells or hyphal elements. The size is  $3.5 \times 4.5$  microns. (Plate 3, Figs. 1, 2.)

*Cultural Characters.*—On glucose agar the streak is filiform, slightly raised, glistening, smooth, and chalk-white. The growth is slow and there is little change in old cultures. There is a filiform growth in gelatin stab, with no liquefaction. No pellicle or ring is present in beerwort or in liquid sugar mediums. Giant colonies may be seen in Plate 6, Fig. 17, and in Plate 8, Fig. 20.

*Physiologic Characters.*—There is slight fermentation of glucose, levulose, and sucrose. This occurs only after a week and the production of gas is never over 10 per cent. of the closed arm of the tube. No decided change in acidity occurs in sugar mediums. There is no change in litmus milk.

The culture was isolated from human feces. (Culture 137.101; Type Specimen 5; Type Slide 137.1.)

This species is very similar in many of its characters to Culture 170.101. The latter, however, ferments glucose and levulose very rapidly and completely. Both of these cultures are slow growing, very smooth, and remain white and even-edged in very old cultures. The surface elevation is not so decidedly convex as in most yeasts of the white, glistening type.

## CRYPTOCOCCUS GLABRATUS SP. NOV.

*Morphology.*—Cells in young cultures are oval or elliptical, and fairly uniform in size and shape; in old cultures cells are round, oval, or elliptical, and more variable in form and size. Budding occurs from the ends or shoulders of the oval and elliptical cells. There are no elongated cells or hyphal elements. The size is  $3 \times 4.5$  microns. (Plate 3, Fig. 12.)

*Cultural Characters.*—On glucose agar the streak is filiform, glistening, raised, smooth, and chalk-white. In old cultures the surface remains smooth and the edge entire. There is a slow growth on all solid mediums; liquid mediums remain clear with little evidence of growth, and no pellicle or ring formation is present. A giant colony may be seen in Plate 8, Fig. 21.

*Physiologic Characters.*—There is rapid fermentation of glucose and levulose. Other sugars are not fermented. Litmus milk becomes only slightly alkaline. No decided change in acid reaction occurs in sugar mediums. Gelatin is not liquefied.

The culture was isolated from human feces. (Culture 170.101; Type Specimen 6; Type Slide 170.)

This species differs in few respects from *Cryptococcus ovoideus*. The cells are more elliptical and the fermentation reactions are unlike.

## MYCODERMA MONOSA SP. NOV.

*Morphology.*—Cells in young cultures are elliptical or narrowly elliptical; in old cultures cells are of various forms, predominantly elliptical, with numerous elongated and irregular forms. Rows of elongated cells in old cultures form a false mycelial development. No true septation is observed. Budding occurs from the ends or from shoulders of the young cells. The size is  $2 \times 5.5$  microns. (Plate 3, Figs. 14, 15.)

*Cultural Characters.*—On all agar slants the streak is spreading, dull, white, flat, and becoming gray with age. A heavy dull pellicle is formed within 24-48



hours on all liquid sugar mediums and on beerwort. There is a villous growth along stab in gelatin. Giant colonies may be seen in Plate 7, Fig. 12, and in Plate 8, Fig. 10.

*Physiologic Properties.*—Glucose and levulose ferment readily. There is no change in litmus milk. Sugar mediums, with an original acidity of  $-1$ , become less acid after 1 week.

The culture was isolated from human feces. (Culture 141.101; Type Specimen 7; Type Slide 141.1.)

MYCODERMA RUGOSA SP. NOV.

*Morphology.*—Cells in young cultures are elliptical, oblong, elongated, or somewhat irregular; in old cultures the cells on the surface of the medium are oblong, ovate, or elongated; beneath the surface very long, narrow cells of hyphal character are produced by the elongation of the bud at the distal end of another elongated cell. No septate mycelium is formed. Budding in young cell occurs from end or shoulder. The size is  $3 \times 6.5$  microns. (Plate 3, Figs. 8, 9.)

*Cultural Characters.*—On glucose agar slant the streak is white, dull, and flat, but not spreading; later the surface becomes glistening and decidedly rugose and pitted. Bushy growths may extend downward into the agar at points along the streak. There is a rapid villous development in gelatin stab cultures. A heavy pellicle is formed in sugar mediums and beerwort. Giant colonies are very distinctive. (Plate 7, Fig. 5.)

*Physiologic Characters.*—No sugars are fermented; there is no change in litmus milk.

The culture was isolated from human feces. (Culture 128.1; Type Specimen 8; Type Slide 128.)

This *Mycoderma* is not distinguishable from several other species, for example, *M. cerevisiae*, as far as the morphologic and physiologic characters enumerated are concerned. An examination of photographs of the giant colonies of various *Mycoderma* species revealed the fact that none of these species produce the peculiar rugose-pitted type formed by the foregoing species. The production of such type of growth is not confined to giant colonies on glucose agar, but is present on slants of glucose and beerwort agar.

PARASACCHAROMYCES ASHFORDI SP. NOV.

*Morphology.*—In young cultures cells are round or slightly oval; in old cultures cells are of many forms: oval, elongated, elliptical, round, or irregular; giant cells are common. Septate mycelium develops in gelatin hanging-drop and in old cultures. Budding occurs from any point on the young cells, but usually near the ends of articles in old cultures. The size is  $4.5 \times 5$  microns. (Plate 4, Figs. 4, 5; Plate 5, Figs. 11, 12.)

*Cultural Characters.*—On glucose agar the streak is filiform, raised, glistening, chalk-white and smooth; later the central portion may become rugose or pitted; the edge of the streak may remain entire or may become decidedly filamentous, due to the outward growing hyphal elements under the surface of the medium (Plate 4, Fig. 5a). There is a growth in gelatin stab at first filiform, later it develops scattered, bushy clusters of filaments. In liquid sugar mediums and beerwort a very evident ring formation occurs; no pellicle is present. Giant colonies may be seen in Plate 7, Fig. 8, and in Plate 8, Figs. 7, 8.

*Physiologic Properties.*—It ferments glucose, maltose, and levulose; occasionally sucrose and galactose are fermented. Yeast-water sugar mediums, with an initial acidity of +1, become more alkaline (Table 6, Culture 2.501). Litmus milk is rendered alkaline in 2 weeks, but is not clotted. Gelatin is rarely liquefied.

The culture was isolated from a sprue patient by Dr. B. K. Ashford in Porto Rico. (Culture D; Culture 2.501 was pronounced by Dr. Ashford to be identical with his organism\*; Type Specimen 4; Type Slide 2.5.)

This species strongly resembles the fungus variously called *Oidium albicans*, *Monilia albicans*, and *Endomyces albicans*. Castellani ('16) has, however, reserved the name *Monilia albicans* for a species which always clots milk, and liquefies gelatin. *Monilia albicans*, *Oidium albicans* and *Endomyces albicans* are synonyms, and if Vaillimin's ('99) results are accepted and are of general application to all of these, the correct name for the species is *Endomyces albicans*, since he states that this species forms asci after the manner of other species of the genus *Endomyces*. Since all efforts to develop the perfect stage of the sprue organism, both by Dr. Ashford and myself, ended in failure and since it differs in many of its physiologic characters from the typical *Endomyces albicans*, it has been thought best to give it specific rank rather than to regard it as a variety of *Endomyces albicans*.

#### PARASACCHAROMYCES THOMASI SP. NOV.

*Morphology.*—In young cultures, cells are elliptical or ovate; in old cultures, surface cells are round, oval, elliptical, or elongated; submedial cells form a distinct mycelium mostly by elongation of cells produced by budding. There is occasional septation in gelatin hanging-drop. Budding occurs from ends or shoulders. The size is  $3.5 \times 5$  microns. (Plate 5, Figs. 4, 5.)

*Cultural Characters.*—On glucose agar the streak is, at first, white, glistening, convex, and smooth; later the surface becomes rugose with a decidedly elevated ridge down the center. Beneath the surface of the medium the radiating hyphae form a villous fringe. In beerwort and liquid sugar mediums no pellicle or ring is present. In gelatin-stab cultures the growth is finely villous. Giant colonies in beerwort gelatin are decidedly yellow in color and otherwise very characteristic. (Plate 6, Fig. 4; Plate 8, Fig. 12.)

*Physiologic Properties.*—Slow fermentation of glucose, levulose, and maltose. In litmus milk there is a decided alkaline reaction.

The culture was isolated from human feces. (Culture 147.103; Type Specimen 10; Type Slide 147.)

This species is similar to *Parasaccharomyces Ashfordi* in its physiologic properties. It differs mainly in its morphologic characters and the type of giant colonies produced. The yellow, rugose colony in beerwort gelatin is especially characteristic and easily distinguishes in this species from *P. Ashfordi*.

#### SUMMARY AND CONCLUSIONS

Yeast-like fungi are commonly found in the intestinal tract of man. They are of many species and, for the most part, such types as are commonly present in nature and known as 'wild yeasts.' It is probable that these yeasts are ingested with the food.

\*Letter, Dec. 6, 1916.

The feces of persons suffering from gastro-intestinal disorders of various types do not yield a larger number of yeasts than those from healthy persons. There is no one species commonly present in the intestinal tract of either healthy persons or those suffering from gastro-intestinal troubles.

In a case diagnosed by highly competent clinicians as sprue, yeast-like organisms of a single species and in great numbers were constantly present. This species is the same as the sprue organism isolated by Ashford.

Nonpathogenic yeasts, when fed in mass, pass through the alimentary tract in a living condition and may be found in great numbers in the feces. They do not cause serious inconvenience when fed alone, and are not retained longer than the ingested food.

The sprue organism, when ingested after being in culture for several months, is not able to cause the disease but is retained in the intestinal tract for a longer time than in the case of the nonpathogenic forms.

The pathogenic yeasts, for the most part, are easily distinguished from the yeast-like fungi isolated from the digestive tract. None of the yeasts isolated from healthy persons or from those suffering temporary intestinal disturbances were identical with the pathogenic yeasts studied.

Generic separation among the yeasts which do not form ascospores is based on the presence or absence and relative predominance of a septate mycelial phase in the life history of the organism, together with the type of budding and striking cultural characters. The genera to which the budding asporogenic fungi belong are: *Cryptococcus*, *Parasaccharomyces*, *Pseudosaccharomyces*, *Pseudomonilia*, *Mycoderma*, and *Zymonema*.

On account of the wide morphologic and cultural variations within a single species, the separation of species is based on the combination of morphologic, cultural, and physiologic characters. The necessity of a standard method of procedure similar to that of the bacteriologists for the separation of species is recognized.

The yeast-like fungus associated with sprue is similar in many of its characters to *Endomyces albicans* and is possibly identical with the yeast commonly found causing thrush in children.

The sprue organism (*Parasaccharomyces Ashfordi* sp. nov.) is easily distinguished from the 'wild yeasts' commonly found in the intestinal tract by its reactions in sugar mediums and milk; and cultural characters, such as the peculiar growth in gelatin-stab cultures and the formation of septate mycelium in gelatin hanging-drop cultures. No

one of these characters when taken alone is sufficient to identify the species and any or all of them vary under some conditions.

Fourteen cultures isolated from the intestinal tract were carefully studied and compared with known species. Of these, 11 were evidently different and distinct species, while 3 were sufficiently like *Parasaccharomyces candida* (*Monilia candida* Bon.) to warrant placing them in this species.

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PLATE 3

FIG. 1. *Cryptococcus ovoideus* sp. nov. Cells from a young, beerwort culture.

FIG. 2. *Cryptococcus ovoideus* sp. nov. Cells from an old, glucose agar culture.

FIG. 3. Culture 141.201. a, Elongated cells with groups of buds. From beneath the medium in an old, glucose agar culture. b, Cells from the surface of the medium.

FIG. 4. *Parasaccharomyces candida* comb. nov. Cells from young, beerwort culture (Culture 229).

FIG. 5. *Parasaccharomyces candida* comb. nov. a, Elongated cells developed in old, agar culture beneath the surface of the medium. The attached globular cells have large refractive globules filling most of the cell interior. b, Cells from surface of old culture showing large refractive globules.

FIG. 6. *Saccharomyces hominis* Busse. Cells from young, beerwort culture showing peculiar type of budding.

FIG. 7. *Saccharomyces hominis* Busse. Cells from old culture showing at b the numerous large granules usually present in old cells.

FIG. 8. *Mycoderma rugosa* sp. nov. Budding cells from young, beerwort culture.

FIG. 9. *Mycoderma rugosa* sp. nov. Cells from old agar culture.

FIG. 10. *Pseudosaccharomyces Stevensi* sp. nov. Cells from young, beerwort culture.

FIG. 11. *Pseudosaccharomyces Stevensi* sp. nov. Cells from old agar slant culture.

FIG. 12. *Cryptococcus glabratus* sp. nov. Cells from young, beerwort gelatin culture.

FIG. 13. *Parasaccharomyces candida* comb. nov. Budding cells from young, beerwort culture. (Culture No. 152.)

FIG. 14. *Mycoderma monosa* sp. nov. Cells from young, beerwort culture.

FIG. 15. *Mycoderma monosa* sp. nov. Cells from old, agar culture.

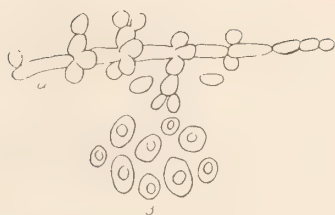
# PLATE 3



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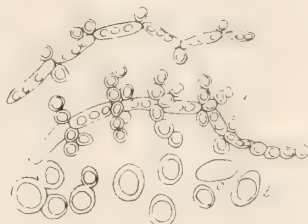
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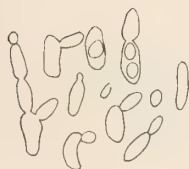
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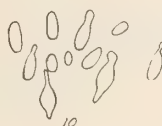
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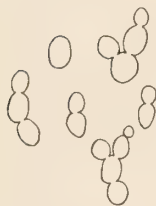
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11



12



13



14



15

PLATE 4

FIG. 1. *Cryptococcus aggregatus* sp. nov. Budding cells from young, beerwort culture.

FIG. 2. *Cryptococcus aggregatus* sp. nov. Cells from old, agar cultures, showing peculiar aggregation of buds about the enlarged, globular cells.

FIG. 3. *Parasaccharomyces candida* comb. nov. a, Portion of septate mycelium from beneath the surface of the medium. b, Cells from the surface of old, agar cultures. (Culture from Centralstelle für Pilzkulturen, Amsterdam.)

FIG. 4. *Parasaccharomyces Ashfordi* sp. nov. Cells from young, beerwort culture. (Culture D from Dr. Ashford.)

FIG. 5. *Parasaccharomyces Ashfordi* sp. nov. a, Moniliform clusters of cells developed beneath the surface of an old agar culture. b, Cells from the surface of the same culture.

FIG. 6. *Parasaccharomyces candida* comb. nov. Young cells from beerwort culture. (Culture from Centralstelle für Pilzkulturen, Amsterdam.)

FIG. 7. *Zygosaccharomyces bisporus* sp. nov. Cells from agar slant culture.

FIG. 8. *Zygosaccharomyces bisporus* sp. nov. a, Typical form of cells from old, agar slant. b, Conjugating cells which do not develop ascospores. c, Ascospore development without conjugation. d, Ascospore development as a result of conjugation. b, c, and d from carrot slant.

FIG. 9. Young cells of Culture 138.102.

FIG. 10. *Saccharomyces ellipsoideus* Hansen. From young culture.

FIG. 11. *Saccharomyces ellipsoideus* Hansen. From old culture.

FIG. 12. Old cells of Culture 138.102.

# PLATE 4

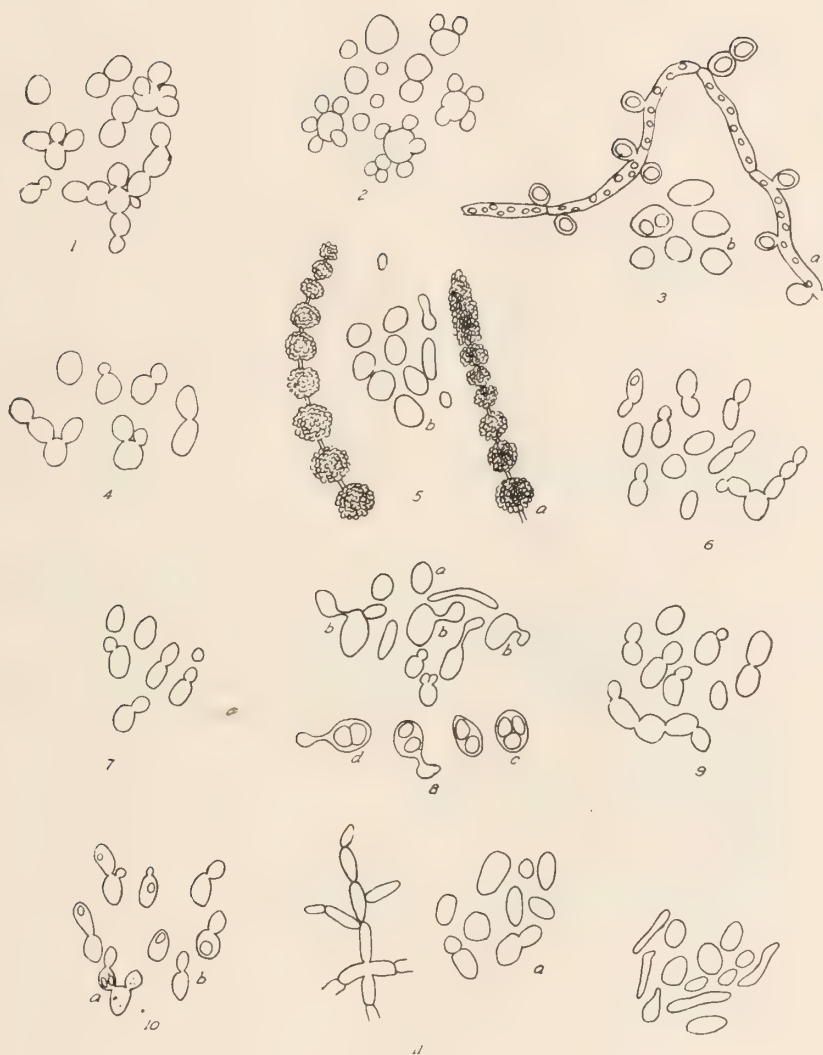




PLATE 5

FIG. 1. *Cryptococcus verrucosus* sp. nov. Budding cells from a young, beerwort culture.

FIG. 2. *Cryptococcus verrucosus* sp. nov. From old, agar culture showing the pronounced granular condition.

FIG. 3. Culture 141.201. Cells from young, beerwort culture.

FIG. 4. *Parasaccharomyces Thomasi* sp. nov. Cells from young, beerwort culture.

FIG. 5. *Parasaccharomyces Thomasi* sp. nov. a, Elongated cells forming a false mycelium beneath the surface of the agar slant. The elongated cells are formed by the elongation of terminal buds. b, Cells from surface of same culture.

FIG. 6. *Parasaccharomyces candida* comb. nov. (Culture 158).

FIG. 7. Culture 141.102. Cells from young, beerwort culture.

FIG. 8. *Oidium albicans*. Cells from young, beerwort culture (Culture from St. Thomas Hospital, London).

FIG. 9. *Oidium albicans*. Cells from surface of old, agar culture.

FIG. 10. Group of yeast cells from a culture of *Parasaccharomyces Ashfordi* showing the great variation in form and size that occurs in a single field of the microscope.

FIG. 11. *Parasaccharomyces Ashfordi* sp. nov. Young cells from Culture 2.501.

FIG. 12. *Parasaccharomyces Ashfordi* sp. nov. Old cells from surface of agar streak of Culture 2.501.

FIG. 13. Septation in the hyphae of *Parasaccharomyces candida*. From gelatin hanging-drop of Culture 152.

# PLATE 5

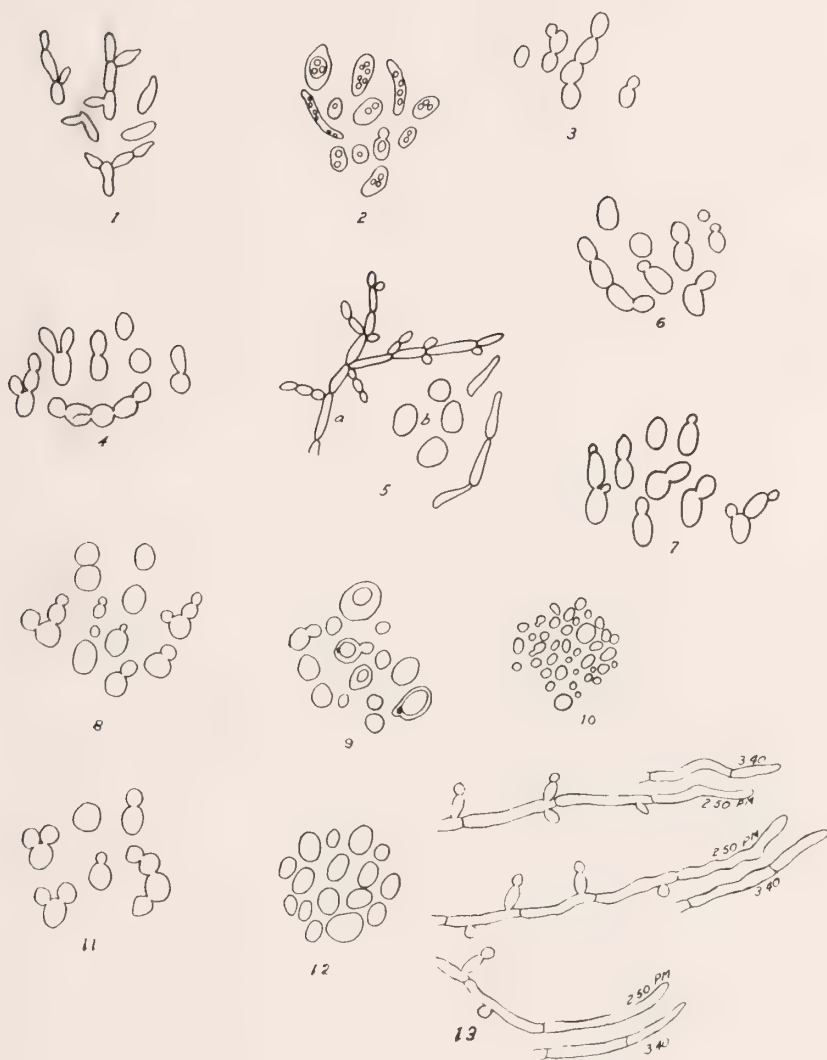


PLATE 6

FIG. 1. Giant colony of Culture 10.102 on glucose agar.

FIG. 2. Giant colony of Culture 141.102 on glucose agar.

FIG. 3. Giant colony of *Parasaccharomyces candida* comb. nov. on dextrose agar. (Culture 229.)

FIG. 4. Giant colony of *Parasaccharomyces Thomasi* sp. nov. on glucose agar.

FIG. 5. Giant colony of Culture 166.102 on glucose agar.

FIG. 6. Giant colony of *Zygosaccharomyces bisporus* sp. nov. on glucose agar.

FIG. 7. Giant colony of Culture 167 on glucose agar.

FIG. 8. Giant colony of Culture 139.201 on glucose agar.

FIG. 9. Giant colony of *Pseudosaccharomyces Stevensi* sp. nov. on glucose agar.

FIG. 10. Giant colony of *Cryptococcus verrucosus* sp. nov. on glucose agar.

FIG. 11. Giant colony of Culture 105, on glucose agar.

FIG. 12. Giant colony of *Cryptococcus aggregatus* sp. nov. on glucose agar.

FIG. 13. Giant colony of Culture 147.102 on glucose agar. This is one of the pink types of yeasts.

FIG. 14. Giant colony of *Oidium albicans* on glucose agar. The rugose region between the inner and outer ring became more broken a few days after this photograph was taken.

FIG. 15. Giant colony of Culture 239.101 on glucose agar.

FIG. 16. Giant colony of Culture 11.901 on glucose agar.

FIG. 17. Giant colony of *Cryptococcus ovoideus* sp. nov. on glucose agar.

PLATE 6

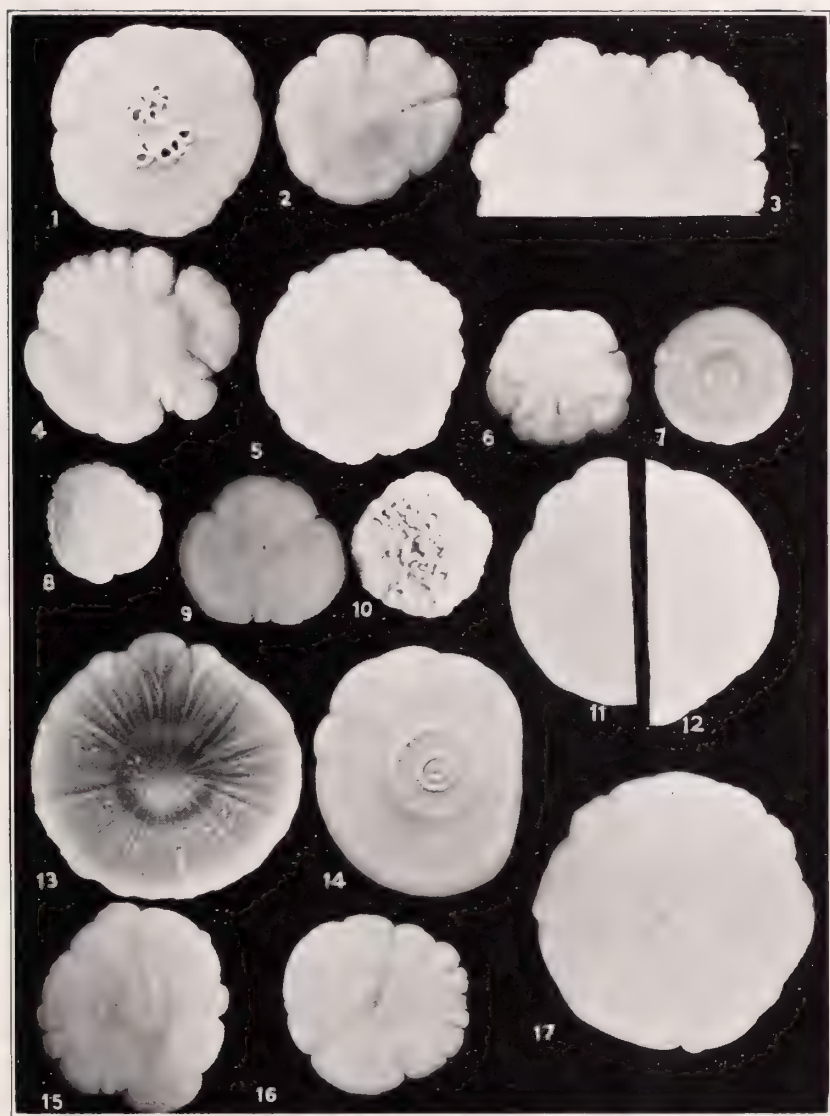


PLATE 7

FIG. 1. Giant colony of Culture 138.101 on glucose agar.

FIG. 2. Giant colony of Culture 139.101 on glucose agar.

FIG. 3. Giant colony of Culture 146 on glucose agar.

FIG. 4. Giant colony of *Saccharomyces cerevisiae* Meyen on glucose agar.

FIG. 5. Giant colony of *Mycoderma rugosa* sp. nov. on glucose agar.

FIG. 6. Giant colony of *Parasaccharomyces candida* comb. nov. on glucose agar. (Culture 152.)

FIG. 7. Giant colony of Culture 11.701 on glucose agar.

FIG. 8. Giant colony of *Parasaccharomyces Ashfordi* sp. nov. on glucose agar. (Culture 2.501.)

FIG. 9. Giant colony of Culture 215.101 on glucose agar. (Probably *Cryptococcus aggregatus*.)

FIG. 10. Giant colony of Culture 42 on glucose agar.

FIG. 11. Giant colony of Culture 120.102 on glucose agar.

FIG. 12. Giant colony of *Mycoderma monosa* sp. nov. on glucose agar.



PLATE 7

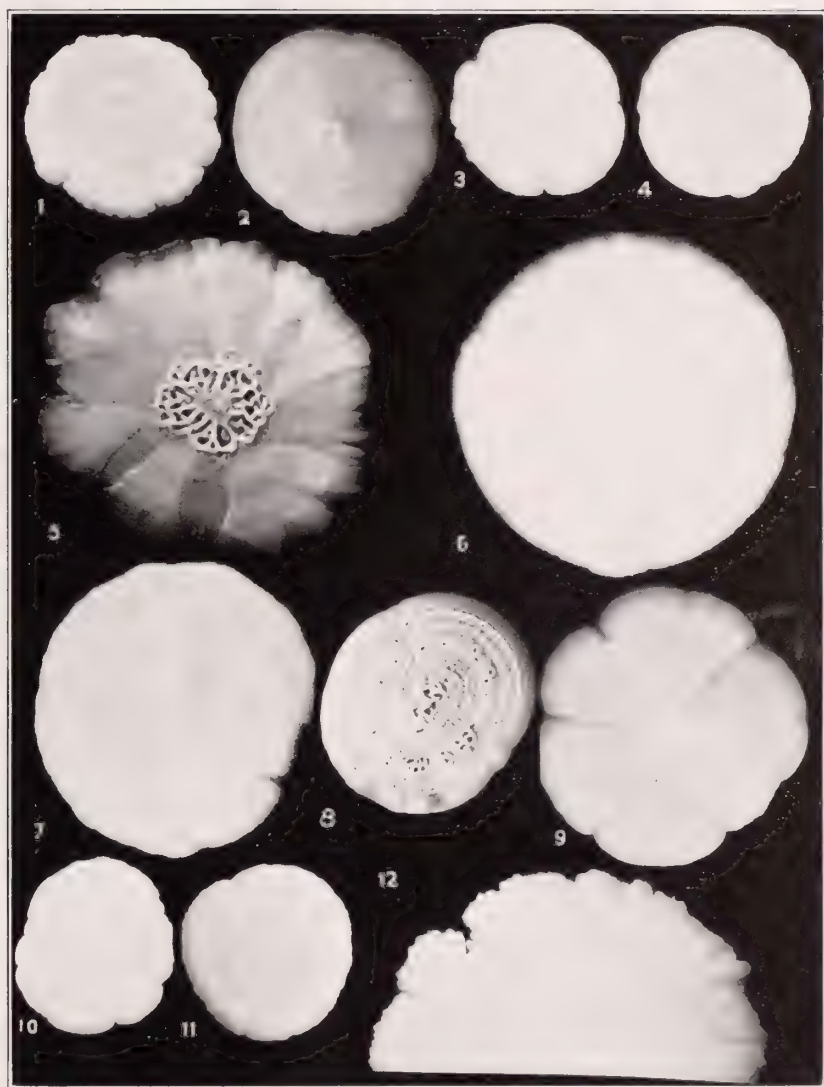


PLATE 8

FIG. 1. Giant colony of Culture 231.102 on glucose agar.

FIG. 2. Giant colony of Culture 11.401 on glucose agar.

FIG. 3. Petri dish showing appearance of plate after inoculation from feces of person fed with yeasts. The white areas at points of contact are yeast colonies. The 4 indefinite light areas are colonies of *Oidium lactis*.

FIG. 4. Giant colony of *Parasaccharomyces Ashfordi* sp. nov. on glucose agar. (From Culture 2.501.) This photograph is taken from another series of cultures from that of Plate 5, Figure 8. The 2 colonies have many characters in common.

FIG. 5. Giant colony of Culture 129.101 on glucose agar.

FIG. 6. Giant colony of *Oidium albicans* on beerwort gelatin. One month old.

FIG. 7. Giant colony of *Parasaccharomyces Ashfordi* sp. nov. on beerwort gelatin. (From Culture 2.501.)

FIG. 8. Giant colony of *Parasaccharomyces Ashfordi* sp. nov. on beerwort gelatin. (From Culture D of Ashford.)

FIG. 9. Giant colony of *Parasaccharomyces candida* comb. nov. on beerwort gelatin. (From Culture 158.) \*

FIG. 10. Giant colony of *Mycoderma monosa* sp. nov. on beerwort gelatin.

FIG. 11. Giant colony of *Parasaccharomyces candida* comb. nov. on beerwort gelatin. (From Culture 152.)

FIG. 12. Giant colony of *Parasaccharomyces Thomasi* sp. nov. on beerwort gelatin. \*

FIG. 13. Giant colony of Culture 138.102 on beerwort gelatin.

FIG. 14. Giant colony of *Cryptococcus verrucosus* sp. nov. on beerwort gelatin.

FIG. 15. Giant colony of Culture 229.101 on beerwort gelatin. \*

FIG. 16. Giant colony of *Saccharomyces ellipsoideus* Hansen on beerwort gelatin. \*

FIG. 17. Giant colony of Culture 141.201 on beerwort gelatin.

FIG. 18. Giant colony of *Pseudosaccharomyces Stevensi* sp. nov. on beerwort gelatin.

FIG. 19. Giant colony of *Cryptococcus aggregatus* sp. nov. on beerwort gelatin.

FIG. 20. Giant colony of *Cryptococcus ovoideus* sp. nov. on beerwort gelatin.

FIG. 21. Giant colony of *Cryptococcus glabratus* sp. nov. on beerwort gelatin.

FIG. 22. Giant colony of *Parasaccharomyces candida* comb. nov. beerwort gelatin. (Culture from Centralstelle für Pilzkulturen.)

FIG. 23. Giant colony of *Mycoderma rugosa* sp. nov. on beerwort gelatin. (Culture 10 days old.) \*

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\* Upside-down.

PLATE 8





## A STABLE POLLEN ANTIGEN \*

RALPH OAKLEY CLOCK

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The early work on the preparation of pollen extracts<sup>1</sup> brought to light the fact that aqueous extracts are unstable and rapidly deteriorate, losing their potency after about 3 weeks. This point is mentioned by Dunbar,<sup>2</sup> and especially emphasized by Koessler,<sup>3</sup> who states:

The pollen extract is not stable, especially not the higher dilutions. By progressing proteolysis, after 3 to 4 weeks, it acquires marked toxic properties which lead to severe reactions. The solution must, therefore, be freshly prepared every 8 to 10 days if these reactions are to be avoided. . . . The material to be injected must be not only sterile, but constantly of uniform potency if used in the same dilution. No extract of pollen can comply with this demand if it is older than three weeks.

It appeared, therefore, that extracts of pollen could not be widely used by physicians unless some method of extraction could be devised which would insure a uniformly stable product. With this end in view, we have prepared pollen antigens which we have subjected to rigid tests for stability.

Pollen from the flowering plants was obtained in the same manner, beginning May 23, 1914, and continuing until Sept. 9, 1914. The plants were closely observed and, as soon as some of the pollen pods began to open, the flowering tops were cut off with suitable stems so that the cut flowers could be easily placed with the cut end of the stem in water, the flower hanging over the edge of the basin. The basins were set in well-lighted rooms, carefully protected from currents of air, on long tables covered with clean smooth paper. As the flowering tops, which continued to 'live' for a short period, began to drop pollen on the paper, the pollen was carefully collected, with camel's hair brushes and dried in thin layers in vacuum dessicators over sulphuric acid, at room temperature, in the dark. The pollen was allowed to remain in the dessicators for a month or more, when it was collected in bulk in glass jars, which were stored in dessicators. In this dry state the pollen does not deteriorate: according to Goodale,<sup>4</sup> it has been kept 25-30 years without any loss in antigenic power. All antigens have been freshly prepared from this stock of dried pollen.

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<sup>1</sup> Lancet, 1911, p. 1572. Ibid., 1911, 2, p. 814. Proc. Soc. Exper. Biol. and Med., 1913, 10, p. 70.

<sup>2</sup> Jour. Hyg., 1913, 13, p. 105.

<sup>3</sup> Forchheimer's Therapeutics of Internal Diseases, 1914, 5, p. 671.

<sup>4</sup> Boston Med. and Surg. Jour., 1915, 173, p. 42.



A mixture was made of equal parts by weight of the pollens of timothy, red top, June grass, orchard grass, rye, sorrel dock, daisy, maize, ragweed, and goldenrod; these being the pollens which are known to cause the greatest number of cases of the vernal and the autumnal type of hay-fever.

Since, in the experimental work reported by others<sup>3</sup> in this country, the pollen was extracted in salt solution, we first prepared antigens after this method in order to test for stability.

*Antigen 10.*—On Jan. 29, 1915, 2.03 gm. of the pollen mixture mentioned were ground with glass dust in a mortar, using 9% salt solution to moisten the mixture which was ground for 2½ hours. A sufficient amount of 9% salt solution was added to bring the total volume up to 145 c.c., which according to Koessler, Noon, Freeman, and others gives a dilution so that 1 c.c. is equivalent to approximately 14,000 units of pollen: the pollen unit having been arbitrarily chosen by those workers as the equivalent of one-millionth gm. of pollen. The mixture was shaken for 30 minutes and placed at 37 C. for 16 hours. It was then shaken for 1 hour, centrifugalized, and passed through Buchner and Berkefeld filters. Trikresol was added to 0.3% as a preservative.

After being prepared, the antigen was kept in an ice-box at about 5 C. Before being titrated, a 10% dilution of the antigen was made using sterile distilled water as diluent, thus making the antigen isotonic, and then further diluted to 1% with physiologic salt solution.

Using the same technic for complement fixation titrations as that adopted by the Research Laboratories of the Department of Health, New York City, 1 pollen unit was found to be equivalent approximately to 1/25 of a unit of Antigen 10 (Table 1), taking a unit of antigen as the smallest amount that gives complete fixation in the hemolytic series. Immune serum was obtained from rabbits which had been immunized with a gradually increasing number of units of pollen. Table 1 shows that 0.000025 gm. of pollen is the antigen unit (smallest amount that gives complete fixation). Since the pollen unit is taken as 0.000001 gm. of pollen, it follows from this table that 1 pollen unit is equivalent to 1/25 of a unit of antigen. With this as a basis for measuring the strength of the antigen, pollen extracted in 9% salt solution gave an antigen that deteriorated quite rapidly. Within 16 days, 40% disintegrated; at the end of 1 month, about 50%; while at the end of 7 weeks, only traces of antigenic properties remained (Table 2).

Eleven antigens were prepared and tested as outlined, but all showed the same rapid deterioration. This confirmed Koessler's statement that the saline extracts are unstable.

<sup>3</sup>Forchheimer's *Therapeutics of Internal Diseases*, 1914, 5, p. 671. *Jour. Am. Med. Assn.*, 1914, 63, p. 141.

Inasmuch as glycerol has been successfully used for many years to preserve the standard diphtheria antitoxin, it was decided to incorporate glycerol in the method of preparing pollen antigens in order to prevent, if possible, the rapid deterioration of pollen protein. Accordingly, the next antigen was prepared by extracting in salt and glycerol as follows:

*Antigen 12.*—On Feb. 26, 1915, 2.98 gm. of the dried pollen mixture already described were extracted with 213 c.c. of a diluent composed of 33⅓% saturated sodium chlorid solution and 66⅔% glycerol. Essentially the same technic was

TABLE 1  
TITRATION OF ANTIGEN 10 (SALINE EXTRACT) FEBRUARY 3, 1915

Number of Tube	Immune Serum, C.c.	Antigen		10% Com-plement, C.c.	0.9% Salt Solution, C.c.	Sensitized Erythrocyte Suspension, C.c.	Results
		1:100 C.c.	Grams of Pollen				
1	0.01	0.25	0.000025	0.1	0	0.2	++++
2	0.01	0.20	0.000020	0.1	0	0.2	+++
3	0.01	0.15	0.000015	0.1	0.05	0.2	++
4	0.01	0.10	0.000010	0.1	0.10	0.2	—
5	0.01	0.05	0.000005	0.1	0.15	0.2	—
6	0.01	0.025	0.0000025	0.1	0.20	0.2	—
7	0	0.4		0.1	0	0.2	—
8	0	0.3		0.1	0	0.2	—
9	0	0.2		0.1	0.05	0.2	—
10	0	0.1		0.1	0.10	0.2	—
11	0	0.05		0.1	0.15	0.2	—
12	0.02	0		0.1	0.20	0.2	—

In these tables, Citron's standard for the strength of a reaction is used: namely, complete absence of hemolysis is indicated by a 4-plus sign (++++); faint hemolysis is shown by a 3-plus sign (+++); partial hemolysis is shown by a 2-plus sign (++); nearly complete hemolysis is indicated by a single plus sign (+); doubtful binding of complement or practically complete hemolysis is shown by a plus and minus sign (±); while a minus sign (—) indicates complete hemolysis.

The mixtures of immune serum, antigen, and complement were put in the ice-box for 15 hours before adding the sensitized erythrocytes. The results were read after 1 hour at 37 C.

TABLE 2  
RESULTS OF FURTHER TITRATION OF ANTIGEN 10

Date of Titration	Antigen		Results
	1-10 C.c.	Grams of Pollen	
Feb. 19, 1915.....	0.25	0.000025	++
	0.20	0.00002	+
	0.15	0.000015	—
March 1, 1915.....	0.25	0.000025	+
	0.20	0.000020	—
March 24, 1915.....	0.25	0.000025	±
	0.20	0.000020	—

followed as described for Antigen 10, except that no preservative was added after the antigen was passed through a Berkefeld filter.

The antigen was stored in the ice-box at 5 C., and before being titrated was rendered isotonic by being diluted to 10% with sterile distilled water, and a further dilution to 1% was made with salt solution.

Complement-fixation titrations, using the technic described, showed that 1 pollen unit was equivalent approximately to  $\frac{1}{20}$  of a unit of Antigen 12 (Table 3). With this as a basis for measuring the strength of the antigen, pollen extracted in 66 $\frac{2}{3}$ % glycerol and 33 $\frac{1}{3}$ % saturated sodium chlorid solution gave an antigen that has proved to be remarkably stable and potent and which remains sterile.

Table 3 shows that 0.000020 gm. of pollen is the antigen unit (smallest amount that gives complete fixation). Since 0.000001 gm. is taken as the pollen unit, it is evident that 1 pollen unit is equivalent to  $\frac{1}{20}$  of a unit of Antigen 12.

TABLE 3  
TITRATION OF ANTIGEN 12 (GLYCEROL-SALINE EXTRACT) MARCH 1, 1915

Antigen		Results
1:100 C.c.	Grains of Pollen	
0.25	0.000025	++++
0.20	0.000020	++++
0.15	0.000015	+++
0.10	0.000010	++
0.05	0.000005	—
0.025	0.0000025	—

By comparing this table with Table 1, it will be seen that the saline extract (Antigen 10) gave complete fixation in the 1st tube only ( $\frac{1}{25}$  of a unit of the antigen equals 1 pollen unit); while the glycerol-saline extract (Antigen 12) gave complete fixation in the 2nd tube also ( $\frac{1}{20}$  of a unit of antigen equals 1 pollen unit). Therefore, the glycerol-saline extract is thus shown to be 25% stronger in antigenic power than the saline extract.

Antigen 12 was stored at ice-box temperature, and was subsequently titrated on the following dates:

March 24, 1915  
 May 6, 1915  
 May 19, 1915  
 June 23, 1915  
 July 19, 1915  
 Sept. 13, 1915  
 May 1, 1916

The results of these titrations were exactly like those shown in Table 3. In other words, the antigen remained for 14 months without any deterioration which could be determined by the complement-fixation

test. During that period, anticomplementary properties did not develop. Repeated bacteriologic examinations showed the antigen to be sterile at all times.

From time to time, many other antigens were prepared and tested in the same manner, and all of them exhibited the same uniform stability.

#### SUMMARY

Pollen antigens, prepared by extracting the dried pollen in  $66\frac{2}{3}\%$  glycerol and  $33\frac{1}{3}\%$  saturated sodium chlorid solution as herein described, and stored in the ice-box at 5 C., remained without any detectable loss in antigenic properties for 14 months.

Such pollen antigens not only remained remarkably stable, but also were protected from bacterial growth, and did not develop anticomplementary properties.

Pollen extracted in glycerol and salt, as herein described, produced antigens that were 25% stronger in antigenic properties than antigens prepared by extracting the pollen in salt solution only.

Pollen extracted in salt solution gave antigens that deteriorated quite rapidly, only traces of antigenic properties remaining at the end of 7 weeks.

# FOOD ACCESSORY FACTORS (VITAMINS) IN BACTERIAL CULTURE WITH ESPECIAL REFERENCE TO HEMOPHILIC BACILLI I\*

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The importance of food accessory substances or vitamins for the proper nourishment and growth of animals has come to be generally recognized. Through the work of Eykmaan it has been clearly shown that beriberi is caused by eating polished rice and can be cured or prevented by adding rice polishings to the food. As shown by Holst and others scurvy no doubt is a disease of a similar character due to the absence in the diet of certain substances found in fresh vegetables, fresh fruits, or fresh animal food. Several other both clinical and experimental diseases probably belong in this group. As far as the higher animals are concerned, the work of Hopkins, Funk, Osborne, and Mendel, McCollum, Moore, and Jackson, and others has now placed these deficiency diseases firmly on an experimental basis, and has shown clearly the necessity of accessory food bodies for proper development and for the maintenance of health. We may now regard these as essential food substances with proteins, carbohydrates, fats, and salts.

Evidence now indicates that the nutrition of the lower animal forms also may be dependent on somewhat similar specific substances. Loeb and Northrop<sup>1</sup> have pointed out that the fly, *drosophila*, requires yeast for its development. They succeeded in cultivating sterile flies and found that they grew and metamorphosed normally on sterilized baker's yeast and water. Mediums containing various salts and many proteins and amino-acids all failed to raise a single fly or pupa. They make the following concluding remarks:

Yeast was then the only medium on which normal larvae could be raised with these sterile cultures of flies, and it seems to be the indispensable food for these insects. Yeast must therefore contain some substance required for their growth and this substance must be rather resistant to heat since yeast heated for one hour at 120 C. is an excellent culture medium. We tried to isolate this substance

\* Received for publication June 6, 1917.

<sup>1</sup> Jour. Biol. Chem., 1916, 27, p. 309.



from the yeast. Yeast extracted with boiling alcohol for 48 hours or with cold alcohol for 10 days was no longer able to serve as food. A mixture of the extracted yeast and of the extractive was no longer adequate to raise the larvae indicating an alteration of the necessary substance by the alcohol. The presence of traces of alcohol in this mixture in itself was not harmful since these flies grow normally in the presence of 2 to 3 per cent. alcohol.

The necessary substance in yeast cannot be salts, or cane or grape sugar since non-sterile flies grow normally on Pasteur media, as Loeb's previous experiments have shown.

The addition of butter, nucleic acid, thymus or thyroid extract to the synthetic culture media mentioned in the table was also without effect. This fact, as well as the fact that the flies will not develop on sterile milk, shows that the necessary substance must be different from that needed for pigeons, rats, and other warm-blooded animals.

It is evident from this statement that the 'substance' in yeast on which the life of this fly depends, while its existence can scarcely be doubted, is, however, indefinite, and its properties and mode of action are quite obscure. It may, I think, quite properly be included in the group of food accessory substances.

The work of Bottomley has given almost equally important results in connection with the growth and development of the higher plants. Bottomley studied this question from the standpoint of soil fertility. He found that peat when inoculated with the nitrogen-fixing organism, *Azotobacter*, and placed in the air for a certain length of time and at a suitable temperature, contained not only certain food substances like potash, phosphates and organic substances, but also certain specific substances which had a powerful effect in inducing plant growth. These substances he was able to isolate from extracts of this 'bacterized' peat by methods quite like those used by Funk and others in obtaining animal vitamins from grains. When the crystalline bodies were added to soil in exceedingly small amounts, green plants planted therein grew and gained in weight much more rapidly than control plants. These substances Bottomley thinks are formed in the peat and also in ordinary soil under the influence of the nitrogen-fixing organisms, and to such bodies he has given the name 'auximones.' In order to detect the amount of these auximones in a given soil he uses a certain soil organism which readily produces a rich scum on an extract of soil containing them. He recommends the bacterized peat as an excellent fertilizer. This work has been confirmed by Jones<sup>2</sup> in this country who showed that heavy application of peat (10%) gave more than 100% increase of plant (radishes) by weight. The expense of preparation, however, may prevent its general application.

<sup>2</sup> Abstracts of Bacteriology, 1917, 1, p. 43.

The multiplicity of food accessory factors is generally admitted by investigators. The vitamins necessary to protect against beriberi are probably different from those necessary to protect against scurvy. Those concerned in the growth and development of the animal also seem to be multiple. Apparently certain ones of them are concerned with the growth, while others control the nutrition, and possibly still others determine the ability of the animal to bear young. Many investigators are accustomed to differentiate the power of foods to perform these various functions, this differentiation probably being based on the accessory food factors, or being somehow related to them.

In this respect the work of McCollum and his associates is important. Based on his many experiments in the feeding of animals he<sup>3</sup> divides the accessory food substances into 2 kinds: the fat soluble substance A and the water soluble substance B. The former is found among other places in butter fats, yolk of egg, meat, and impure lactose. The latter occurs especially in grains and generally in foodstuffs of vegetable and animal origin. Here he would include the vitamins of beriberi. He doubts the existence of more than 2 of these accessory food substances in any food, and makes the suggestion that scurvy is caused by intestinal derangement or stasis. As causes of faulty nutrition he enumerates in addition to these 2 food factors, a 3rd factor which concerns the inadequacy of the protein content because of an unsatisfactory yield of amino-acids, a 4th relating to the proper inorganic content of the food mixture, a 5th attributable to toxic substances in some natural foodstuffs like cotton seed and wheat products, and perhaps also a 6th factor concerned with the mechanical action of foods on the digestive tract, causing distention or feces of an unfavorable character.

Important points concerning the food accessory factors may be here briefly summarized. These bodies apparently comprise a large group of substances having certain properties in common but in many respects being quite different. A certain specificity seems to exist. The beriberi vitamin will not prevent scurvy, nor will certain substances that will cure or prevent scurvy have any effect on beriberi. They all seem to act in small amounts or in very high dilutions. Toward heat they are more or less tolerant, but in this respect considerable variation seems to exist. Some appear to withstand the boiling temperature for some time, others apparently do not. Some

<sup>3</sup> Jour. Am. Med. Assn., 1917, 68, p. 1379.

are water soluble and others are fat soluble. These substances may vary in their physiologic functions in that some seem to affect or control growth, whereas others are chiefly concerned with ordinary processes of waste and repair. Whether they are used or could serve as food alone is not known, but they seem to act as substances accessory to the other food constituents. Their mode or mechanism of action is not understood and their chemical nature is not known.

A few years ago Dr. Moore and I undertook some experimental work in connection with the scurvy-like disease in guinea-pigs produced with milk, grains, and other foods. Since that time I have been interested in studying the question of the possible rôle that similar bodies might play in the growth of certain bacteria. The review of the rôle that these substances play in the nutrition of both the higher and lower animal forms, and also in higher plants would lead one to believe, *a priori*, that examples of the activity of similar substances might appear in bacterial nutrition.

This paper deals largely with the hemophilic bacteria. Pfeiffer<sup>4</sup> in 1892 described the influenza bacillus and in studying its properties made the discovery not only that blood was necessary for its continued growth, but also that hemoglobin was the essential constituent in the blood on which growth depended. This he did by obtaining the hemoglobin in pure crystalline form and adding it to ordinary mediums. These observations have been confirmed by many workers (Huber, Voges, Cantani, Ghon and Preyss and Luerssen), including myself. Serum and other constituents of the blood are not essential.

Pfeiffer did not make quantitative determinations as to the amount of hemoglobin necessary for growth. Several years ago while attempting to throw light on the mechanism of the action of hemoglobin in bacterial culture I<sup>5</sup> determined that hemoglobin was necessary in only very small amounts for the growth of hemophilic bacilli. In mediums containing as little as 1:180,000 of hemoglobin the organism may continue to grow. Strains and mediums vary somewhat. In many of the experiments the dilution point of growth extinction was lower, being reached at dilutions 1:10,000-1:100,000. It was largely on this account that at that time I suggested that hemoglobin acted in the rôle of a catalytic agent in bacterial culture.

Czaplewski<sup>6</sup> also pointed out the small amount of hemoglobin that is necessary for the growth of the influenza bacilli. He used an

<sup>4</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1893, 13, p. 357.

<sup>5</sup> Jour. Infect. Dis., 1907, 4, p. 73.

<sup>6</sup> Centralbl. f. Bakteriol., I, O., 1902, 32, p. 667.

amount of blood that hardly tinted the medium, and obtained with such dilutions even better growth than on the mediums containing large amounts of blood, such as Pfeiffer first used. I have also noted this fact that a small amount of blood is often preferable to larger amounts in growing these organisms. Czaplewski did not make definite quantitative determinations of the amount of hemoglobin necessary for growth. Ghon and Preyss<sup>7</sup> likewise noted that an extremely small amount of hemoglobin suffices for the growth of the influenza bacillus, stating that amounts of hemoglobin not demonstrable with the spectroscope are still sufficient for this purpose.

Another point of importance is that hemoglobin, either in low or high concentration alone will not support the growth of these bacteria. This was pointed out by Ghon and Preyss<sup>8</sup> and also by Luerssen<sup>9</sup>, who noted that hemoglobin in water agar is not a suitable medium. Even with the addition of peptone no growth will occur. By adding broth or milk to the hemoglobin growth will take place and continue indefinitely on transfer. I have recently gone over this work testing the growth of several strains of influenza bacilli in hemoglobin solutions from the dog, ox, sheep, human, cat, pig, and chicken. The solutions of hemoglobin used in this work were prepared by Dr. W. H. Welker of the Department of Physiology and Physiological Chemistry, University of Illinois, to whom I wish here to express my obligations. The hemoglobin crystals were added directly to water plus 0.5% sodium chlorid, to 2% water agar, to peptone water agar, and to ordinary plain agar. The bacilli continue to grow only in the last mentioned mediums. It would seem evident from these observations that the rôle of hemoglobin is not primarily that of a nutritive substance. I refer here only to the hemophilic bacteria, of course, since many other bacteria grow well in water solutions of pure hemoglobin or in all the above mentioned mediums.

It was noted by Pfeiffer that if blood-agar tubes were heated for 1 hour at 70 C. the hemophilic bacilli still readily grew on them, and he also found that hemoglobin coagulated by boiling would still support an appreciable growth though not a luxuriant one. From this observation he concluded that it was the iron content of the hemoglobin and not its oxygen-carrying power which in some way made possible the development of these organisms. He associated this

<sup>7</sup> *Centralbl. f. Bakteriol.*, 1902, 32, p. 96.

<sup>8</sup> *Ibid.*, 35, p. 531.

<sup>9</sup> *Ibid.*, p. 434.



phenomenon with the growth of other bacteria known as iron bacteria in water-containing iron. Ghon and Preyss<sup>7</sup> studied the relation of heat to hemoglobin and came to the conclusion that heat, even boiling temperature or above, did not destroy the power of the hemoglobin to stimulate growth. I have made similar observations and noted that heating even in the autoclave will not totally destroy this property of hemoglobin. I may point out that in order to obtain appreciable growth one must have in the mediums the particles of coagulated hemoglobin. If the brownish coagula are filtered out or are allowed to settle out the medium will yield little or no growth. When such a solution of coagulated hemoglobin is smeared on agar and the medium inoculated the growth is seen to occur immediately about the coagulated particles, but not elsewhere. With such solutions of coagulated hemoglobin, while growth does occur, it is as a rule not as profuse as with the unheated hemoglobin, unless another organism is grown in symbiosis with the hemophilic bacillus. This symbiotic relationship I shall discuss presently. We must conclude, therefore, that coagulation by heat and heating even above the boiling point does not entirely destroy the power of hemoglobin to support the growth of these bacteria.

To sum up then the characteristics of hemoglobin in relation to the growth of hemophilic bacteria we may state that (1) hemoglobin is essential for their growth; (2) it is sufficient in high dilution; (3) alone, it will not support growth, other proteins being necessary for continued development, and (4) it does not lose its power of supporting growth, at least not entirely, through prolonged heating at the boiling point or even higher. On analyzing these points one is struck by the close correspondence between them and the properties and mode of action, as far as is known, of the food accessory factors in the higher forms of life. Further discussion of these points will appear.

In addition to the hemoglobin factor, another factor enters in the growth of hemophilic bacilli. It was noted first by Grassberger<sup>10</sup> that when these bacteria were grown on hemoglobin mediums, if mixed with other organisms, the growth was decidedly more abundant. He notes this phenomenon especially about staphylococcus colonies, but other organisms produce the same effect. It was this observation which first gave rise to the common practice of inoculating

<sup>10</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 453.



the entire surface of a slant with influenza bacilli and then making a staphylococcus streak through the center. If on blood plates sown with influenza bacilli other organisms are grown, large colonies of the former will appear about the latter, producing a striking appearance and suggesting a satellite relation. This influence extends to a distance of 2-4 m. or more about the foreign colony. There seems to be little difference as to what organism is used as a companion for the influenza bacillus. I have tested many different organisms in this regard but they all give quite uniform results. Staphylococci, streptococci, both hemolytic and nonhemolytic, pneumococci, meningococci, diphtheria bacilli, various chromogens, blastomycetes, sporothricha, yeasts, and many others all show this phenomenon with influenza bacilli. It does not depend on hemolysis since both hemolyzers and nonhemolyzers manifest it. Nor is it dependent on reaction since both acid- and alkali-producers show it.

I have made a thorough search for substances which might behave like these bacterial colonies, using for this purpose a hemoglobin-agar plate rather thickly sown with influenza bacilli. In addition to bacteria, pieces of fresh animal tissue show this phenomenon. Kidney, liver, spleen, brain, myocardium, testicle, lung, and muscle of the rabbit and guinea-pig were tested, and all yielded quite uniform results. A drop of fresh blood on the surface of the blood-agar plates shows little or no such activity. This stimulation of growth does not depend on the hemoglobin in the tissues. A small piece of the inside of a potato or carrot placed on the surface also shows this symbiotic effect exactly like the animal tissues. It is needless to state that all such tissues must be obtained under sterile precautions, since other bacteria would produce the same result. If one heats the pieces of animal or plant tissues to 60 C. for 30 minutes they still manifest this stimulating action. When heated to boiling, however, for 15 minutes the tissues, both plant and animal, lose this power. Killed bacteria also lose to some extent but not entirely their influence on such hemoglobin plates. The animal tissues, since they contain hemoglobin, when placed on plain agar plates sown with influenza bacilli, support growth of these bacilli as one would expect. However, carrot or potato tissue, unheated or heated, when added to plain agar, yield no growth whatever. Animal substances free from hemoglobin, like spermatic fluid, yolk or white of egg, spinal fluid, ascites fluid, etc., will not support growth consistently, but on sterile sputum and bile mediums I have obtained definite growth for 1 or 2 generations. In mixed cultures

also influenza bacilli may grow on nonhemoglobin mediums for some time. Neisser<sup>11</sup> claimed that he grew them in mixed cultures with *B. xerosis* for 20 generations in plain agar. I have grown them with *B. diphtheriae* on plain agar through 6 generations. Ordinarily, however, they soon disappear from the mixed cultures after a few transfers.

Ghon and Preyss have explained this growth on plain mediums with other organisms, no doubt correctly, by showing that plain mediums often may contain appreciable quantities of hematin, which, when associated with other bacteria, will support the growth of hemophilic bacilli. Hematin, however, alone, unlike hemoglobin, added to mediums will not permit their growth; only when it is mixed with bacteria will it exercise its power in this respect. These investigators noted also that either living or dead bacteria, killed by heat or otherwise, when associated with hematin would cause abundant growth of influenza bacilli. Either substance alone would not do this. The hematin which they used was prepared by digesting acidified hemoglobin with pepsin for several weeks, and the deposit purified by filtration, washing, and extraction with ether. This solution keeps for a long time. A suspension of bacteria may also be prepared and plain agar to which the bacteria and hematin are added, they claim, makes a very satisfactory medium for continued cultivation of the organisms. I have tested hematin agar with and without bacteria and can confirm these results. Hematin alone in mediums will not yield growth; with bacteria good growth takes place.

It seems then that in plain agar hemoglobin alone will yield growth, but when it is associated with bacteria or tissues, either plant or animal, especially if alive, there occurs what we may designate an optimum growth. Plant and animal tissues and bacteria by themselves will not sustain growth. It appears, therefore, that there is some factor or factors in the latter tissues which somehow act with the hemoglobin to favor growth. Hematin alone will not sustain growth, but associated with bacteria it will do so.

It is interesting to note here the close correspondence that seems to exist between these 2 growth factors in bacterial culture, namely, hemoglobin and the tissue factor, and the 2 substances detected by McCollum and his associates in their diet experiments with animals. McCollum differentiated, as already stated, 2 unidentified

<sup>11</sup> Deutsch. med. Wchnschr., 1903, 29, p. 462.

dietary factors, a fat soluble substance A and a water soluble substance B, and describes them thus :

One (substance B) is soluble in water and alcohol and apparently never associated with the lipoids, when these are isolated from natural food stuffs, the other (substance A) soluble in fats. The latter is extracted from milk, egg yolk, kidney and probably other animal tissues by ether but is not removed by this solvent from either the seeds or the leaves of plants. The former is universally present in foodstuffs of vegetable and animal origin but is absent or nearly so from crystalline sugar, starch, and fats, and is present in but small amounts in polished rice and probably on those foods derived from the endosperm of seeds by milling processes. Where the reaction of the medium is alkaline, strong heating of foods probably leads to its destruction.

It is evident, however, that the substances A and B of McCollum are different in many respects from the substances necessary for bacterial culture, but in general it may be said they are analogous or may indeed be placed together in groups with certain common properties. For example, hemoglobin is a water and alcohol soluble substance and may possibly be grouped with the B substance of McCollum. The other factor in influenzal culture is closely associated with other bacteria and living or fresh animal and plant tissue. The question may be asked whether this factor or substance might possess in general the properties of substance A, which according to McCollum is found in the kidney and probably other tissues, in milk, eggs, etc.

The properties of the 2d substance in influenzal culture as yet have not been studied very carefully. This is being carried out at present along lines which may permit us to compare them with the other accessory substances in diets of higher animals. Ghon and Preyss found that ash of staphylococci would not behave as did the dead or living bacteria with hematin. The substance, therefore, is organic. Whether it resides in the bacterial body or results from the action of the bacteria or tissues on the mediums is not known. It may be doubted, too, whether one and the same substance is responsible for the symbiotic effect of bacteria or tissue in hemoglobin mediums and the satellite phenomenon observed on hematin agar. There seems to be some difference in the resistance to heat of the substances concerned in this phenomenon. This point should be studied further. So far as the 2d substance is concerned then we know perhaps even less about its nature and mode of action than we do about the vitamins or the substances of McCollum, though its existence and influence can not be doubted.

Assuming that hemoglobin behaves as a food accessory substance in bacterial nutrition it is possible that through a study of its action we may be able to throw light on the mechanism of the activity of food accessory substances in general. We know much more about the chemistry and the function of hemoglobin than we do about vitamins. However, at the present moment, we cannot say with any certainty how hemoglobin acts in supporting the growth of hemophils. In the body the chief function of hemoglobin is to carry oxygen, but it evidently does not serve in this capacity for bacteria, since coagulating and heating does not destroy its usefulness for the latter, but does destroy its oxygen-carrying function. Furthermore, hemocyanin,<sup>3</sup> a copper-containing substances in the blood of many lower animals, performs the same function as an oxygen-carrier as hemoglobin but does not support the growth of hemophils. It is not poisonous to them for when hemoglobin is added to the mediums containing hemocyanin the organisms proceed to develop normally. Pfeiffer thought the iron of the hemoglobin was the essential substance, and this is the opinion of Luerssen and Ghon and Preyss. The latter, who made the most careful study of this point, believe that the iron must exist in an organic form readily obtainable and that other bacteria by their reducing action make the iron of the hemoglobin and hematin available for the hemophilic bacteria. It should be stated that various iron salts and organic preparations of iron will not serve in place of hemoglobin. Hematogen, ferratin, and many other preparations have been tested with negative results. Cantani and Ghon and Preyss found that bile from certain persons, but not from all, would behave much as hematin behaves. Bile pigments and salts gave negative results. Since bile from different persons varies in its iron-content these results may depend on the iron-content, though this point was not determined by them. Ghon and Preyss after trying many iron preparations succeeded in getting some growth of influenza bacteria with foreign organism by adding to ordinary mediums an iron preparation made by dissolving iron hydroxid in hydrocyanic acid, and sterilizing by boiling. They state that when this was added in small amounts to agar, with foreign organisms, growth of the hemophils regularly occurred about the latter, though the colonies were indeed small. The point should be emphasized, however, that hemoglobin is the only substance known which alone supports the growth of these bacteria. All other iron-containing substances, such as hematin, require the aid of foreign cells.



Recently Dorothy Lloyd<sup>12</sup> in England has called attention to the rôle of vitamins in the growth of the meningococcus. She concludes from the study that the primary cultivation of the meningococcus *in vitro* is only possible in the presence of certain accessory growth factors present in blood, serum, milk, and other animal fluids, and probably present also in vegetable tissues. These accessory factors are bodies which are moderately heat-stable, and are soluble in water and alcohol. They are rapidly adsorbed from solution by filter paper, but do not appear to be adsorbed by glass wool. On account of an inverse relationship between the amount of amino-acid present in the medium and the amount of vitamins required to stimulate the growth of laboratory strains it is suggested that the action of the accessory growth factors is to increase the reaction velocity of the proteolytic metabolism of the meningococcus. The meningococcus after isolation from the body gradually develops a change in its metabolism of such a nature that it becomes increasingly independent of a vitamin supply on the medium. Old laboratory strains need no additional vitamin supply if the medium contains an abundant supply of free amino-acids. She was able to obtain the stimulating factors from fresh blood by extracting it with 80% alcohol. Evidently the substance with which she is dealing is closely related to the accessory factors in the diet of animals.

The question naturally arises as to the rôle of such substances in the growth of other varieties of bacteria and especially the pathogenic forms in the body. We are familiar with the important rôle that blood, body fluids, fresh animal tissue, and undenatured proteins play in the cultivation of many varieties. The suggestion seems reasonable that 1 of the determining factors for such growth may be the vitamin-content of the tissues for specific kinds of organisms. This subject, therefore, at once becomes related to problems of virulence, susceptibility, and immunity.

#### SUMMARY

Food accessory factors are now recognized for higher animals, for certain lower forms, for the higher plants, and for bacteria.

These substances are necessary for proper nutrition in addition to proteins, fats, carbohydrates, and salts.

They act in very small amounts and probably by themselves, and, at least in case of certain bacteria, are not able to support growth. They are relatively resistant to heat.

<sup>12</sup> Jour. Path. and Bacteriol., 1916, 21, p. 113.



For the growth of hemophilic bacteria pure hemoglobin is 1 factor and a 2d factor resides in foreign bacteria and fresh animal and plant tissue. To obtain maximum development both factors are necessary. Hematin alone does not support growth but does so in conjunction with other bacteria, living or dead. The similarity of the action of these 2 factors to the action of the fat soluble substance A and the water soluble substance B of McCollum is pointed out.

The mechanism of the action of such substances is not clear. The availability of iron in the hemoglobin may be the determining factor in the growth of hemophils. The 2d factor or substance may act by rendering this element more available.

The multiplicity of food accessory factors is recognized by most investigators. Their mode of activity may be as varied. Their action in connection with hemophilic bacteria seems to center around the metabolism of iron.

The suggestion may be made, in conclusion, that the activity of these substances in animals and higher plants may concern or somehow control the metabolism of certain elements like iron, phosphorus, calcium, or iodine, as well as possibly the protein metabolism.

## PRESERVATION OF ANTISHEEP HEMOLYTIC AMBOCEPTOR IN GLYCEROL \*

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In the complement-fixation test for the diagnosis of syphilis, gonorrhea, whooping cough, etc., it is usual to employ a hemolytic amboceptor which, although kept in a sterile manner, slowly deteriorates after several months even when stored in the ice-box.

The practical advantage of a suitably preserved and stable hemolytic amboceptor lies in the fact that it does not require retitration or standardization at different periods. There is also the advantage of the prevention of contamination, thereby prolonging the usefulness of the amboceptor.

To produce so stable an amboceptor necessitated the use of some suitable agent which would inhibit bacterial growth and chemical changes, thus preventing the immune bodies in the serum from deteriorating, and which, at the same time, would in no way interfere with the test to be conducted.

Tricresol, phenol, and chloroform were tried, but were discarded because they caused a precipitate when added to the serum in sufficient quantities to destroy bacteria, and they exerted no preservative power on the immune bodies contained in the serum. Furthermore, serum preserved with any of these chemical agents, after an indefinite period, often becomes anticomplementary so that it cannot longer be used.

The bactericidal properties of glycerol have been demonstrated by its use in the preparation of glycerolated vaccine virus; while its power to prevent molecular changes (due to its hygroscopic properties), thereby preventing deterioration, has been proved by its effective use in holding the standard diphtheria antitoxin, sent out by the United States government, unaltered for long periods. It was, therefore, decided to try glycerol as a preservative for antisheep hemolytic amboceptor. This investigation was begun on Feb. 7, 1914. Subse-

\* Received for publication June 1, 1917.

quently, Ruediger<sup>1</sup> reported glycerol to be an ideal preservative for human serum intended for the Wassermann reaction. He also found that phenol, tricresol, and chloroform were not suitable for this purpose.

# PREPARATION OF THE SERUM

Rabbits—large Belgian hares give the best result—immunized against washed sheep erythrocytes were anesthetized and bled from the carotid artery. The blood was collected in a sterile manner in small test tubes. As soon as the blood began to clot, the tubes were placed in the incubator for 1 or 2 hours. The clots were loosened from the wall of the tubes by means of a sterile wire, and the tubes then placed in the ice-box at 5 C. for 12-18 hours. The clear serum was pipetted off and inactivated at 55 C. for one-half hour. It was then mixed with an equal volume of sterilized, neutral glycerol and stored in small rubber-stoppered bottles. Repeated bacteriologic examinations carried out for 3 years have shown the sera preserved in this manner to be sterile at all times.

# TITRATIONS

Antisheep hemolytic amboceptors, thus prepared, were titrated at intervals of 1-2 months during 3 years, using one tenth of the volume of the classic Wassermann reaction. As controls, similar titrations were made of serums preserved with other chemical agents, as well as serum without preservative.

TABLE 1  
TITRATION OF GLYCEROLATED AND OTHER SERUMS

Amboceptor No.	Preservative	Date of Preparation	Date of Titration	Titer	Date of Titration	Titer	Date of Titration	Titer
121	0.8% Tricresol	7/ 7/13	7/22/13	1:2000	2/25/14	1:1000	2/23/17	Anticomplementary
123	0.5% Phenol	8/14/13	8/15/13	1:4000	2/25/14	1:1000	2/23/17	Anticomplementary
124	0.3% Chloroform	9/27/13	9/29/13	1:4000	2/25/14	1:1000	2/23/17	Anticomplementary
132A	None	2/ 7/14	2/25/14	1:5000	6/15/14	1:3000	2/23/17	Contaminated and anticomplementary
132B	50% Glycerol*	2/ 7/14	2/25/14	1:5000	6/15/14	1:5000	2/23/17	1:5000
133A	50% Glycerol after which the mixture was heated to 55 C. for one-half hour	2/ 7/14	2/25/14	Anticomplementary	6/15/14	Anticomplementary	2/23/17	Anticomplementary
133B	50% Glycerol*	2/ 7/14	2/25/14	1:2000	6/15/14	1:2000	2/23/17	1:2000
141	50% Glycerol*	2/16/14	2/25/14	1:3000	6/15/14	1:3000	2/23/17	1:3000

\* In the preparation of these amboceptors, glycerol was added after\*the serums had been inactivated at 55 C. for one-half hour.

<sup>1</sup> Philippine Jour. Sc., Sect. B, 1916, 11, p. 1.

Serums 132 and 133 were divided into 2 portions, A and B. No preservative was used in 132 A, but 132 B was preserved in glycerol as described. Serum 133 A was mixed with an equal volume of glycerol, and then heated to 55 C. for one-half hour; while the other portion, 133 B, was preserved in glycerol, according to the method described.

As will be seen from Table 1, only the serums which were preserved in 50% glycerol retained their original titer after 3 years; and further, such serums did not develop anticomplementary properties nor become contaminated.

#### ANTICOMPLEMENTARY PROPERTIES

If the serum was first mixed with glycerol, and the glycerolated serum then heated to 55 C. for one-half hour (as was done in the case of 133 A), the serum was rendered anticomplementary. The anticomplementary properties thus produced could not be destroyed by repeated heating at any time during the 3-year period. As pointed out by Ruediger,<sup>2</sup> in order to prevent glycerolated serum from becoming anticomplementary, the serum must be inactivated at 55 C. for one-half hour before being mixed with glycerol.

#### COMPLEMENT-FIXATION TESTS

Antibody-content titrations were made with several antigens and known positive sera, using 141 and 132 B amboceptors (preserved in glycerol) in order to determine what effect, if any, the glycerolated amboceptor had on the reaction. In order to have adequate controls, duplicate tests were made with amboceptor 132 A and an amboceptor (250) obtained from the New York City Department of Health, which latter contained no preservative; in this respect 132 A and 250 were similar except that they were prepared in different laboratories.

Neutral extracts of pure cultures of gonococci, meningococci, and streptococci were used as antigens; also saline extracts of pollen. One fourth of the anticomplementary dose was used in each tube.

The serums of guinea-pigs were used in a 10% dilution of physiologic salt solution as complement.

A 5% suspension in physiologic salt solution of washed, sedimented sheep corpuscles was used.

<sup>2</sup> Philippine Jour. Sc., Sect. B, 1916, 11, No. 2, p. 87.

TABLE 2  
 RESULTS OF COMPLEMENT-FIXATION TESTS\*

Antigen and Dilution	Amboceptor	Amount of Antigonococcus Serum, C.c.								Serum and Antigen Controls
		0.001	0.0008	0.0006	0.0005	0.0004	0.0003	0.0002	0.0001	
Gonococcus.... 1:10	141†	++++	++++	++++	+++	++	+	—	—	—
	132B†	++++	++++	++++	+++	++	+	—	—	—
	132A†	++++	++++	++++	+++	++	+	—	—	—
	250‡	++++	++++	++++	+++	++	+	—	—	—
Meningococcus 1:30		Amount of Antimeningococcus Serum, C.c.								
		0.001	0.0008	0.0006	0.0005	0.0004	0.0003	0.0002	0.0001	
		141†	++++	++++	++++	+++	++	—	—	
		132B†	++++	++++	++++	+++	++	—	—	
Streptococcus. 1:5	132A†	++++	++++	++++	++++	+++	++	—	—	—
	250‡	++++	++++	++++	++++	+++	++	+	—	—
Pollen..... 1:100		Amount of Antistreptococcus Serum, C.c.								
		0.01	0.008	0.006	0.005	0.004	0.003	0.002	0.001	
		141†	++++	++++	++++	+++	++	—	—	
		132B†	++++	++++	++++	+++	++	—	—	
Pollen..... 1:100	132A†	++++	++++	++++	++++	+++	++	—	—	—
	250‡	++++	++++	++++	++++	+++	++	+	—	—
		Amount of Antipollen Serum, C.c.								
		0.01	0.005	0.004	0.003	0.002	0.001	0.0009	0.0008	
		141†	++++	++++	++++	+++	++	+	—	
		132B†	++++	++++	++++	+++	++	+	—	
	132A†	++++	++++	++++	++++	+++	++	+	—	—
	250‡	++++	++++	++++	++++	+++	++	+	—	—

\* In this table, Citron's standard for the strength of a reaction is used: namely, complete absence of hemolysis is indicated by a 4 plus sign (++++); faint hemolysis is shown by a 3 plus sign (+++); partial hemolysis is shown by a 2 plus sign (++); nearly complete hemolysis is indicated by a single plus sign (+); while a minus sign (—) indicates complete hemolysis.

† These amboceptors were preserved in glycerol.

‡ No preservative was used in these amboceptors.

The serums from horses actively immunized against cultures of gonococci, meningococci, and streptococci were employed; also serum from rabbits immunized against pollen. Each of these serums was titrated against its homologous antigen.

As is shown in Table 2, glycerol in the amboceptor serum (141 and 132 B) did not interfere with the complement-fixation reaction in the slightest, the same degree of fixation resulting when a glycerolated amboceptor was used as when one without glycerol was used.



## SUMMARY

1. Fresh antish sheep hemolytic amboceptors that were heated to 55 C. for one-half hour, and then mixed with an equal volume of glycerol did not deteriorate, but retained their original titer for 3 years. During that period, anticomplementary properties did not develop.

2. The glycerol in the glycerolated antish sheep hemolytic amboceptor did not influence the complement-fixation reaction.

3. Fresh antish sheep hemolytic amboceptors that were inactivated and then preserved in glycerol, as herein described, were not only remarkably stable but were also protected from bacterial growth for a period of 3 years.

# STUDIES ON PNEUMOCOCCI AND STREPTOCOCCI \*

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## REVIEW OF LITERATURE

The differentiation of the organisms of the pneumococcus-streptococcus group is of importance in the interpretation of blood-culture results, and in the etiologic study of certain infectious diseases, especially endocarditis and rheumatic fever.

The separation of pneumococci from streptococci was first made on a morphologic basis. The tendency of the pneumococcus to grow in pairs of lanceolate cocci surrounded by a well-defined capsule, distinguished it from the streptococcus, which grew in longer or shorter chains of ovoid or flattened cocci without a capsule. Special methods for the detailed study of capsules were devised by Welch, Hiss,<sup>25, 26</sup> Buerger,<sup>8-10, 13, 14</sup> Wadsworth, Rosenow, Medalia, and others. Buerger found that many streptococci encountered in routine work had capsules. He recognized certain types of capsules which he considered diagnostic for pneumococci and *Streptococcus mucosus*. A comparative study of the various capsular stains by Baehr and Kantor<sup>3</sup> lead to the conclusion that Buerger's method is the most satisfactory and reliable for diagnostic purposes, being succeeded in value by those of Welch and Rosenow.

In 1901, Libman<sup>38</sup> published his observations on the phenomenon of precipitation by streptococci when grown on glucose-serum agar, and the failure of pneumococci to produce this effect. A little later Hiss<sup>25</sup> published his studies on the serum-water medium of Hanna<sup>22</sup> plus inulin, which pneumococci fermented and streptococci did not ferment. The medium of Hanna consisted of 1 part serum to 9 of water, while that of Hiss consists of 1 part serum to 2 of water. Buerger<sup>11</sup> improved this medium, rendering the results more constant and decisive by the addition of 2% Witte's peptone.

Neufeld<sup>50</sup> observed that pneumococci were dissolved in rabbit bile. Levy<sup>37</sup> found that sodium taurocholate dissolves pneumococci and the *Streptococcus mucosus* grown in broth, but not streptococci. Mandel-

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baum<sup>49</sup> found that ox bile was suitable for the test. Libman and Rosenthal,<sup>42</sup> in 1908, confirmed the differential value of the bile test, and the correctness of the classification of the organisms in use in the laboratory.

Several varieties of streptococci were observed in the many pathologic conditions caused by the chained cocci, and various classifications were introduced. Thus Fehleisen and Rosenbach distinguished the streptococci of erysipelas from those of ordinary infections by certain differences exhibited in the ordinary fluid and solid mediums. Von Lingelsheim distinguished between *Streptococcus longus* and *Streptococcus brevis*, depending on the length of the chains, the former supposedly pathogenic, the latter saprophytic. It is now generally conceded that the streptococcus of erysipelas and the streptococcus of ordinary cellulitis are identical, and that the classification based on the length of chains is not at all satisfactory.

In 1903, Schottmüller<sup>65</sup> made an important addition to the cultural methods of differentiation by studying the growth of the organism on plates of human-blood agar. He divided streptococci into 3 groups, thus:

1. *Streptococcus longus* seu *erysipelatos*, virulent organisms growing in long chains and producing hemolysis on blood medium.
2. *Streptococcus mitior* seu *viridans*, less virulent organisms growing in shorter but often in long chains, and producing green non-hemolyzing colonies in plates of blood agar.
3. *Streptococcus mucosus*, an organism more closely related to the pneumococcus, producing a transparent mucoid growth with intense green coloration of the blood agar.

Schottmüller found *Streptococcus mitior* in a variety of conditions, the most important being a group of 7 cases of endocarditis of a chronic infective type which he later designated as "Endocarditis lenta."<sup>66</sup> Similar clinical pictures had been described by Lenhartz,<sup>36</sup> Litten,<sup>44-46</sup> and Harbitz.<sup>23</sup> Lenhartz called the causative agent "*Streptococcus parvus*"; Litten called it "*Streptococcus gracilis*." Schottmüller showed that distinctions of size and delicateness of growth were insufficient to characterize an organism of this group, and introduced the blood-plate method of differentiation. In 1909, Rosenow<sup>54</sup> described cases of "chronic septic endocarditis" from the blood of which he obtained organisms he looked on as altered pneumococci. In 1910, Libman and Celler<sup>41</sup> published "The Etiology of Subacute

Infective Endocarditis," in which the cultural characteristics of the organisms causing this clinical entity were fully described and compared with those of pneumococci, hemolytic streptococci, and *Streptococcus mucosus*, from which they were clearly distinguishable.

The English school, represented by Gordon,<sup>21</sup> and Andrewes and Horder,<sup>2</sup> made extensive studies on the fermentative properties of streptococci from various sources, and classified them according to the source and the carbohydrates they fermented. Thus they described *Streptococcus equinus*, *Streptococcus mitis* (in saliva and feces), *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus anginosus*, *Streptococcus fecalis*, and pneumococcus. Horder,<sup>32</sup> reported 88 cases of subacute and 18 cases of chronic bacterial endocarditis, in which the causative coccus was classified as *Streptococcus salivarius* or *Streptococcus fecalis*. Rarely the influenza bacillus or the pneumococcus was the cause. The pneumococcus cases are doubtful as they had pneumonic complications, and, as Horder said, the infection was most likely a terminal one. Buerger<sup>15</sup> made a critical study of the English biochemical method of classification, using the mediums suggested by Libman as being the optimum for the purpose, namely, fluid mediums containing carbohydrate and unheated serum.\* He concluded that "no hard and fast differences in fermentative properties characterize the various groups, the authors preferring to have recourse to the most frequent habitat and to chemical tests. They have failed to use the most favorable medium for growth of the organisms."

An extensive study by Hopkins and Lang,<sup>31</sup> using fermentation methods, yielded an unsatisfactory classification into pathogenic and saprophytic types of streptococci, the latter, however, including organisms from cases of endocarditis. The authors employed mediums without the use of serum and hence their fermentation results are not so reliable as those of Holman.<sup>28,30</sup> The latter, using optimum serum-broth medium for his fermentation tests, and preferring, for valid reasons, qualitative to quantitative tests, classified both hemolytic and anhemolytic streptococci into 8 groups each. The carbohydrates used were lactose, mannite, salicin, and inulin. Studies on capsules and precipitation were, unfortunately, not made. His nomenclature corresponds closely with that of Andrewes and Horder.

\* Studies by Libman, Celler, and Sophian (results presented to the American Association of Pathologists and Bacteriologists in 1913, not yet published) demonstrated conclusively that broths containing carbohydrates and unheated serum constitute the best mediums for the study of acid-production, being far superior to the serum-water mediums suggested by Hanna and Hiss. The serum-water mediums plus peptone give decidedly better results than those without peptone, but are distinctly inferior to the mediums mentioned.

The subject of rheumatic streptococci and of the variation and mutation of organisms will be dealt with later.

Studies in agglutination,\* conglutination, precipitins, opsonins, complement fixation, and anaphylactic reactions, while yielding interesting phenomena have not shown sufficiently definite or constant results to be of practical value in the differentiation of the organisms.

#### METHOD OF STUDY

While most organisms are readily diagnosed by means of a few tests, not infrequently error thus results. Organisms which seem typical when incompletely studied are often found to be atypical when thoroughly observed. Consequently, much of the older literature on this subject is difficult to interpret, for the organisms have not been described in a manner which makes possible a just comparison with modern work. It is of the greatest importance that any organism obtained from the body should be studied thoroughly at first isolation, for there is evidence to show that residence in the blood stream, localization in new parts of the body, that is, in metastatic foci, cultivation on artificial medium, and animal passage may alter the properties of an organism.

1. *Morphology*.—Gram stains are made on spreads of colonies in the blood-culture plates and of the 24-hour growth on 0.5% glucose-serum agar. The relative size, the shape, and the grouping of the organisms are noted.

2. *Capsule*.—The Buerger<sup>8,9</sup> differential capsular stain is employed. The spread should be made from a fresh culture on a moist slant of 0.5% glucose-serum agar, for this medium is the most favorable for the development and preservation of capsules. We distinguish a streptococcus mucosus type, a pneumococcus type, and atypical forms. Often the capsule is described in detail with special reference to definition, indentation, and staining of the capsular substance.

3. *Glucose Broth 2% (Merck's Peptone)*.—The 24-hour growth is observed for turbidity, granular, clumpy, flocculent or mucoid growth, and sediment. Hanging drops are made to determine the length and type of chains formed.

4. *Precipitation Test*.—This consists in inoculating on a slant of 0.5% glucose-serum agar. The ascitic serum for the test should have a specific gravity of not less than 1.013, and should be added to 3 times the volume of 0.5% glucose agar. The slant must be fresh enough to show condensation water at the bottom of the tube. The culture is incubated 24-48 hours. If the organism precipitates, the clear medium becomes hazy, cloudy, or even opaque white in color. Occasionally the whitening is seen only about the condensation water. This effect on the medium is due, according to Libman,<sup>39</sup> to the precipitation of the serum albumin by the acids, chiefly lactic, formed by the action of the organisms on the glucose

\* The classification of pneumococci into 4 strains by means of agglutination phenomena (Dochez) will not be taken up.



in the medium. The test is one of the most reliable and constant for differentiating streptococci, which precipitate, from pneumococci and *Streptococcus mucosus*, which do not precipitate.

5. *Inulin Fermentation*.—Buerger's<sup>11</sup> modification of the Hiss serum-water 1% inulin medium is used. By the addition of 2% Witte's peptone, more constant and rapid results have been obtained. Tubes are incubated for 4 days, as some organisms ferment the inulin slowly. Some organisms do not ferment the inulin, but cause a decolorization or reduction of the litmus.

6. *Bile Test*.—Two tubes of neutral sugar-free broth containing 1% Witte's peptone are inoculated and incubated until sufficient growth is obtained. To one of these one fifth of the volume of filtered sterile ox bile is added, and the tube is thoroughly shaken. If solution of the growth occurs it usually takes place in a few minutes, but 20 minutes should be allowed, as occasionally a soluble organism may resist the lytic action of the bile for that length of time. The 2nd tube is used for comparison. The objection to this test is the difficulty which is encountered in obtaining sufficient growth of some organisms. The medium used is not a good one for the growth of many of these cocci, and its nutritive properties cannot be enhanced because sugars and serum interfere with the reaction, as shown by Nicolle and Adil Bey. At times it is impossible to perform the test because of insufficient growth. At other times several tubes are inoculated and the sediment from them divided into equal parts for the test.

Libman and Rosenthal<sup>12</sup> found that the lytic action of the bile was not complete, for they obtained growth on blood agar from cultures which had been mixed with bile in 9 pneumococci of 19 tested, and in 3 streptococcus mucosus strains of 12 tested. Of 68 streptococci, none was dissolved, and all gave growth.

7. *Blood-Agar Plates*.—In addition to a study of the appearances of the colonies in the original blood-culture plates, surface inoculations are made with the loop on blood-agar plates with heterologous human blood, and on standard ox-blood plates, prepared according to the method of Bernstein and Epstein.<sup>7</sup> The human blood plates are made in the proportion of 2 c.c. of blood to 15 c.c. of plain agar. Ox-blood plates are made in the proportion of 1 c.c. of defibrinated formalinized ox blood to 10 c.c. of sugar-free agar. A study of the effect of glucose in ox-blood agar plates has shown that the presence of 0.5% and 2% glucose may diminish or entirely inhibit the hemolytic properties of an organism, but may intensify the production of greenish coloration by organisms possessing this property.

The streak cultures on blood plates are observed for 4 days. The manner in which they are examined is of importance, for very different appearances are obtained by direct and by transmitted light. For my observations, the plate was held parallel to and at a distance of about 8 inches from a white background, such as a porcelain slab or a sheet of white paper on the working desk. The eye can look both down on and through the plate in this way. Note is made of the size and shape of colonies, dryness or moisture of growth, color, etc. Some are colorless and transparent, some gray, white, grayish-white, grayish-green, or brownish-green. Changes in the surrounding medium are noted, such as clearing (hemolysis), haziness or precipitation, production of green, or other coloration. Many organisms observed in this way show a linear clearing, which is of no importance. Clearing to be of diagnostic value must be 2-4 mm. in width, and must be produced in 24 hours.

The clearing about a colony was formerly thought to be due to a solution of the hemoglobin of the red corpuscles, and was, therefore,

called hemolysis. Similar appearance, however, is seen about foreign particles in blood plates. It is also to be noted that outside the zone of clearing produced by the growth of a colony is a zone of condensation of the blood pigment, and one is led to believe from these 2 observations that the process may be of a physical nature, leading to a driving away of the blood pigment with a heaping up of it outside the clear zone.\* Thalhimer notes that intact red blood cells in the medium are essential for clearing, because this phenomenon was not observed in hemoglobin-agar medium.

The cause of green coloration in blood plates was investigated by Ruediger.<sup>63</sup> He observed that some streptococci which clear in plain blood agar grow green in glucose blood agar, and that pneumococci, which grow intensely green in 1% glucose blood agar and moderately green in ordinary blood agar, produce only a faint green in sugar-free blood agar. He concluded that the green coloration produced by bacterial colonies in blood-agar plates is dependent on the production of acid and the action of this acid, probably lactic, on the red corpuscles. He accounted for the varying intensity of green produced by a given organism in different bloods by varying sugar content of the bloods used. This theory cannot be reconciled with the facts obtained from the use of the precipitation test, for pneumococci produce less acid but grow green, while streptococci produce more acid but cause clearing, or may at times grow green. Precipitation, it will be remembered, is due to acid-production, and if blood-plate appearances are due to the same cause, then the results of these 2 tests should parallel one another.

That the presence of glucose has a decided effect on clearing and the production of green coloration is a fact which was observed in these studies. In view of the influence of glucose in the medium it is important in blood-plate studies that the agar should be free of this carbohydrate. The use of agar, which is entirely sugar-free as employed in the ox-blood plates, inhibits the production of green coloration by some streptococci, but does not interfere with the typical growth of pneumococci.

Butterfield and Peabody<sup>17</sup> showed that the pneumococcus produces methemoglobin or hematin by its action on blood. Baerthlein<sup>4</sup> found 3 kinds of changes produced by bacteria in blood mediums: (1) Hemolysis, liberation of blood pigment, stromata of corpuscles left intact.

\* In this connection the phenomenon of concentric clearing is of interest (p. 424).

This occurs in fluid mediums only. (2). 'Hemoglobinopepsie,' liberation and digestion of blood pigment, stromata left intact. This occurs in solid mediums and produces partial clearing with slight greenish discoloration. (3) 'Hemopepsie,' complete digestion of pigment and stromata, the medium being completely cleared. This also occurs in solid mediums.\*

Lyall<sup>47</sup> studied the effect of 0.5 c.c. of 18-hour broth cultures incubated for 1 hour with 1 c.c. of 5% washed sheep corpuscles. Those streptococci which caused clearing on blood-agar plates caused complete hemolysis in this test. Those streptococci which produced more or less green coloration on blood plates either caused methemoglobin formation in the cells without solution, or were indifferent in their effect. Krumwiede and Valentine,<sup>35</sup> using this test, did not find a sharp division of anhemolytic streptococci into 2 groups.

Schottmüller<sup>65</sup> in his blood-plate studies, used 2 c.c. of human blood in 5 c.c. of agar, and made both surface and deep inoculations. This is a heavy concentration of blood, and may vitiate some of the results, for I have seen streptococci which caused marked clearing on plates 2:15, fail to produce any clearing on plates of higher blood concentration. Rosenow adds 0.3-0.5 c.c. of defibrinated human or rabbit blood to 5 c.c. of agar. Holman uses 5 c.c. of defirinated human blood to 100 c.c. of agar.

The method of studying the organisms of the pneumococcus-streptococcus group outlined in this section has been gradually developed at the hospital laboratory under the direction of Dr. Libman, and by means of it there is rarely difficulty in assigning a given organism to its proper class.

#### CLASSIFICATION OF THE ORGANISMS

While the majority of organisms belonging to the pneumococcus-streptococcus group, as encountered in routine bacteriologic work, adhere to certain well defined types, many observers have met with individual organisms varying from type in greater or lesser degree. Some of these atypical forms partake of the characters of 2 of the type organisms, and may be regarded as transitional forms in the process of evolution.

\* Note the similarity between (2) and (3) of Baerthlein and Types alpha and beta of Smith and Brown.<sup>68</sup>

The organisms of the pneumococcus-streptococcus group are best divided into 4 types, namely:

1. *Streptococcus mucosus*.
2. *Pneumococcus*.
3. *Streptococcus anhemolyticus*.
4. *Streptococcus hemolyticus*.

Within each group are found variations from type. The 3rd group, in fact, includes such a variety of organisms that a type can hardly be said to exist. This class of cocci has been the cause of much confusion, for it contains bacterial flora found in the mouth, throat, and intestinal tract of man and animals, leading a purely saprophytic existence, and forms culturally indistinguishable from these, but possessing

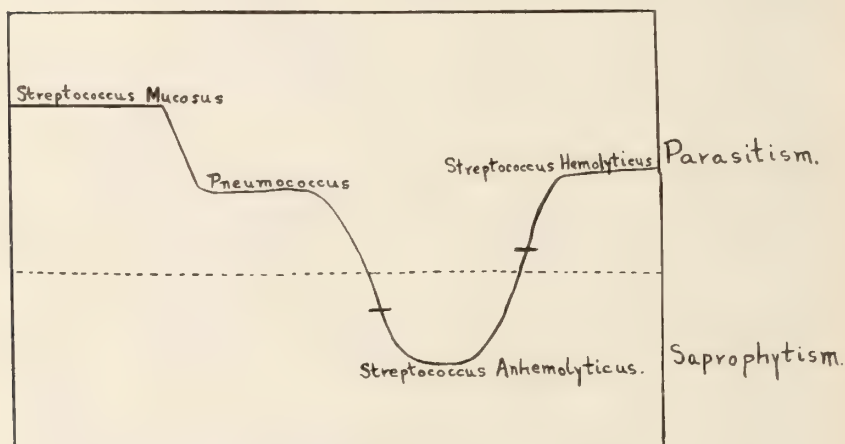


Fig. 1.—Illustrating that there are types and transitions between the types. Note that we have saprophytic pneumococci and parasitic anhemolytic streptococci.

a degrees of pathogenicity for man and animals. While they may be found in localized infections in man, they have seldom been found in the blood stream, except in the disease variously known as subacute and chronic malignant endocarditis, infective or bacterial endocarditis, or endocarditis lenta. The organisms causing this disease have in the course of evolution acquired the property of parasitism. This view was expressed by Andrewes.<sup>1</sup> We may regard such organisms as facultative parasites.

The other 3 groups are far more pathogenic for man and animals. While we meet with them as saprophytes in the mouth, throat, and

nasal cavities, we look on them as having great pathogenic potentialities. They are to be considered as essentially parasitic and only facultatively saprophytic. While they have a greater uniformity and fixation of characters than the anhemolytic streptococci, resulting in the production of type organisms, variants may appear under varying environmental circumstances. Moreover, characters acquired as the result of environmental factors may be lost when the particular exciting circumstances cease to exist.

I, therefore, conceive of all these bacteria as forming a continuous line of organisms which may be represented in a graphic manner (Fig. 1). The division into parasitic and saprophytic forms is, of course, only a relative one, for all these organisms can live a saprophytic existence, and all can acquire parasitic properties, but they have a greater adaptation to one or the other mode of life.

TABLE 1  
TYPE ORGANISMS

	Gram Stain and Morphology	Buerger Capsule Stain	Glucose Broth	Precipitation	Inulin	Bile Test	Blood-Agar Plates
Streptococcus mucosus	+ Round, ovoid, or biscuit shaped; pairs and short chains	Streptococcus mucosus type	Diffuse turbidity	0	+	Dissolved	Mucoid, green
Pneumococcus	+ Lanceolate diplococci	Pneumococcus type	Diffuse turbidity	0	+	Dissolved	Green
Streptococcus anhemolyticus	+ Round, ovoid, lanceolate, bacillary; pairs, chains, groups	1. Pneumococcus type 2. Atypical 3. None	1. Diffuse turbidity 2. Clumps 3. Granular	+	+ or 0	Not dissolved	Green, gray, white, colorless (slight clearing)
Streptococcus hemolyticus	+ Longer or shorter chains of flattened cocci	1. Atypical 2. None	Granular growth	+	0	Not dissolved	Clearing marked

#### TYPE ORGANISMS AND VARIATIONS

In Table 1 are described the morphologic and cultural characters of the 4 groups of organisms when typical. As stated, a type does not exist for the anhemolytic streptococci, but I have given in this table the chief variations met with. This group bridges the gap between the pneumococci and the hemolytic streptococci. In Table 2 are noted all the variations met with when these organisms are isolated from the



blood, the throat, local foci, etc., and when they have been cultivated on artificial medium, both favorable and unfavorable for the preservation of their type characters.

*Streptococcus mucosus* has been fully described in the papers of Howard and Perkins,<sup>33</sup> Schottmüller,<sup>65</sup> and Buerger.<sup>13</sup> This organism as indicated in the graphic scheme and in the table of cultural characters seems much more closely related to the pneumococci than to the streptococci. This view is confirmed by the fact that we encounter organisms which grow mucoid and green on blood plates, are dissolved

TABLE 2  
VARIATIONS FROM TYPE ORGANISMS

	Gram Stain and Morphology	Buerger Cap- sule Stain*	Glucose Broth	Precipi- tation	Inulin
<i>Strepto- coccus mucosus</i>	+ Pairs and chains of twos; round, ovoid, biscuit shaped	1. <i>Streptococcus mucosus</i> type 2. Atypical 3. None	(With serum) Diffuse tur- bidity; mucoid white sediment; fairly long straight chains	0 (rarely posi- tive)	+ (very rarely nega- tive)
<i>Pneumo- coccus</i>	+ Lanceolate diplo- cocci; short chains; round, ovoid and bacil- lary forms	1. <i>Pneumococcus</i> type 2. Atypical 3. None	Diffuse turbidity; diplo- cocci and short chains; at times long chains	0 (rarely posi- tive)	+ occasion- ally nega- tive)
<i>Strepto- coccus anhemo- lyticus</i>	+ Smaller and larger cocci; single, pairs, chains, ir- regular groups; round, ovoid, navicular, lanceo- late and bacil- lary forms	1. <i>Pneumococcus</i> type 2. Atypical 3. None	1. Diffuse turbidity 2. Growth of discrete clumps or colonies 3. Granular growth; diplo- cocci, short, long, con- volved and conglom- erate chains	+ (rarely nega- tive)	+ or 0
<i>Strepto- coccus hemolyti- cus</i>	+ Longer or shorter chains of round, ovoid or flat- tened cocci	1. Atypical 2. None	Granular growth adherent to side of tube; often a flocculent sediment; short, long, convoluted, and conglomerate chains	+	0 (rarely posi- tive)

\* Bold face figures indicate unusual finding.

in bile, ferment inulin, and do not precipitate, but show a large typical pneumococcus capsule. Such organisms have been called mucoid pneumococci,\* and they occupy a position between the typical *Streptococcus mucosus* and the typical pneumococcus. I isolated one such organism from the blood of a case of pneumonia with double empyema and purulent pericarditis.

\* For this reason the name *Pneumococcus mucosus*, which has at times been used for the *Streptococcus mucosus*, has caused confusion and had best be abandoned.

I shall now discuss the diagnostic value of each of the tests employed, and note the variations met with.

*Morphology.*—While one may strongly suspect the identity of an organism freshly isolated from the body when stained by Gram's method, the variations in morphology are so great that no final conclusions can be drawn from observations made in this way. Involution forms are found soon after transplantation, and on prolonged cultivation on artificial medium the whole appearance of an organism in size, shape, and grouping may be altered.

TABLE 2.—*Continued*  
VARIATIONS FROM TYPE ORGANISMS

Bile Test	Blood-Culture Plate Plain Blood Agar 2:15	Blood-Culture Plate 2% Glucose Blood Agar 2:15	Surface Inoculations on Standard Plain Ox-Blood Agar Plates 1:10
Dissolved	Surface colonies mucoid, confluent; deepest colonies spread out between medium and plate in a mucoid fashion; intermediate colonies grayish-green; surrounding medium intensely green	Same appearances but production of green pigmentation more intense	Mucoid confluent growth, surrounding zone of intensely green medium, 2-4 mm. wide
Dissolved	Surface colonies ring form; deep colonies grayish-green, surrounding medium moderately green; rarely clearing or concentric zones of clearing	Same appearances, production of green more intense; at times the medium becomes brownish; no clearing	Grayish-green moist growth; zone of gradually deepening green medium, 2-4 mm. wide
Not dissolved	Gray or white colonies, surrounding medium unchanged, slightly greenish or bull's eye appearance; sometimes a zone of clearing, 1-2 mm. wide in 48 or 72 hr.	Greater tendency to produce green pigmentation of medium; bull's eye appearance less marked; no clearing	1. Green 2. Gray 3. White 4. Colorless 5. Linear clearing
Not dissolved	Gray or white colonies surrounded in 24 hr. by clear zone 2-4 mm. wide; rarely concentric zones of clearing	Zone of clearing narrower; clearing may be altogether absent or delayed	1. White 2. Gray 3. Colorless } Wide zone of clearing of clearing in 24 hr. less

*Capsule.*—Buerger<sup>11</sup> in his work on pneumococci and allied organisms depended for diagnosis in cases of doubt on the capsule stain, because at that time no organism with a pneumococcus type capsule had been encountered which did not on further study prove to be a pneumococcus or an organism which could be made to revert to a pneumococcus. Since that time, however, anhemolytic streptococci have been isolated from the blood of 2 cases of subacute streptococcus endocarditis (by Drs. Robert Levy and E. P. Bernstein) which possessed typical pneumococcus capsules. I have also found typical pneu-

mococcus capsules on anhemolytic streptococci from other sources, among them strains of 'rheumatic' cocci. Therefore, while in the majority of instances a definite diagnosis of an organism can be made by means of the capsular stain alone, in a small number of instances, notably in the case of intermediate and transitional organisms, it is misleading.

The streptococcus-mucosus type capsule appears as a wide, gelatinous, intensely stained envelope around 2, 4 or more cocci. Its edge is poorly defined and hazy; there is no sharp limiting membrane; there are no indentations between the individual organisms. The pneumococcus type capsule is a fairly wide envelope including diplococci or a short chain. Its edge is sharply defined and deeply stained; there is a distinct limiting membrane. The capsular substance does not stain as intensely as the capsular membrane. The latter follows a course parallel to the outline of the included diplococci, and shows distinct indentations between the individual organisms. From 30-45% of hemolytic streptococci possess capsules of varying appearance. Most often there is a narrow close fitting but well defined membranous envelope which may show no indentations or only slight constrictions between pairs of cocci. The narrow zone of capsular substance stains faintly. Again, the capsule may be ill defined and consist of a fairly well stained envelope without limiting membrane or indentations. When the pneumococcus and *Streptococcus mucosus* are grown artificially, for some time their capsules may degenerate and become indistinguishable from those seen on many hemolytic streptococci. A full discussion of the types of and variations in capsules of these organisms, with illustrations, may be found in Buerger's articles. Concerning anhemolytic streptococci, I have found that they may have pneumococcus type capsules, atypical capsules, or no capsules.

The 4 strains of *Streptococcus mucosus* and the 24 strains of pneumococci studied showed type capsules. Of 10 strains of anhemolytic streptococci from the blood of cases of subacute endocarditis, 1 had the pneumococcus type capsule, and 9 were nonencapsulated. Of 12 anhemolytic streptococci from other sources, 2 had the pneumococcus type capsule, 3 had atypical capsules, and 7 had none. Thus, of 22 anhemolytic streptococci, 6, or about 28%, were encapsulated. Of 21 hemolytic streptococci, 7 or about 33%, had atypical capsules. Of 69 streptococci (hemolytic and anhemolytic) studied by Libman and Rosenthal,<sup>42</sup> 32, or about 45%, had capsules. Of 43 streptococci

studied by me (21 hemolytic and 22 anhemolytic) only 13, or about 30%, had capsules.

*Glucose Broth.*—The appearance of the growth in this medium and the type of chain-formation are on the whole of little diagnostic value, for while certain types of organisms behave in a certain way in this medium the variations and exceptions are large in number. Moreover, cultivation on mediums may rapidly abolish the characteristic growth of an organism in fluid medium. *Streptococcus mucosus* and pneumococci produce as a rule a diffuse clouding of the medium, while streptococci grow in a granular manner. The tendency of the cocci from cases of subacute endocarditis to grow in discrete clumps or colonies in broth is a property which, according to Rosenow, is closely associated with their power to produce embolic endocardial lesions in animals. Of the 10 strains I studied, 7 showed this characteristic to a greater or lesser degree on isolation, while 3 strains produced a diffuse turbidity. Of 12 anhemolytic streptococci from other sources, 9 showed a clumpy or granular growth, while 3 produced diffuse turbidity.

*Precipitation.*—Of the differential tests, this is one of the most valuable and constant. It separates sharply *Streptococcus mucosus* and pneumococcus, which produce no whitening on glucose-serum agar, from *Streptococcus anhemolyticus* and *Streptococcus hemolyticus*, which produce whitening. Of the 4 strains of *Streptococcus mucosus* and the 24 strains of pneumococci, none precipitated. Libman and Rosenthal<sup>12</sup> had 2 strains of *Streptococcus mucosus* which precipitated, one for 4 and the other for 2 generations. Two of their pneumococci, which had been in bile, precipitated for 1 generation. No coccus from a case of subacute endocarditis has been encountered which did not precipitate within 48 hours. Of my 10 strains, all precipitated intensely in 24 hours except Organism B, obtained from Dr. E. P. Bernstein, which possessed a pneumococcus type capsule and whose power of precipitation diminished noticeably after several transplantations. Of the 12 anhemolytic streptococci from other sources, Organism 26496 did not precipitate until the 3rd transplantation and passage through a mouse. All the others of this series precipitated. Of the 21 hemolytic streptococci, all were found to precipitate.

*Inulin Fermentation.*—*Streptococcus mucosus* ferments inulin very regularly. Buerger's 16 strains, and the 12 studied by Libman and Rosenthal all fermented inulin. Of the 4 strains I studied, 3 fermented



and 1 did not, even after animal passage. The property of fermenting inulin is not constant for pneumococci, as a given organism may ferment in one generation but not in another. On 1st isolation, however, the property is fairly constant. Of 24 pneumococci, 22 fermented strongly and rapidly; 2 fermented slowly and slightly. Libman and Celler<sup>41</sup> state that about one third of the cocci from cases of subacute endocarditis fermented inulin on isolation and retained the property in spite of prolonged artificial cultivation. Of my 10 strains, 7 fermented, 1 reduced, and 2 had no effect. Dr. Celler tested this property in a series of old stock cultures and found that all strains which had previously fermented inulin still did so, and that 2 strains, previously noted as not fermenting, now did ferment. The oldest of these cultures which fermented was about 5 years old. Of 12 anhemolytic streptococci from sources other than endocarditis cases, 5 fermented inulin. Of these 5, 1 lost the property on 4th transplantation, another fermented slightly, and then not at all 2 months after isolation, a 3rd lost the property on 3rd transplantation, but regained and retained it after passage through a mouse. Of the other 7 strains, 1 caused reduction, while 6 had no effect. Of the 21 hemolytic streptococci, none fermented inulin.

The power of the inulin-fermenting anhemolytic streptococci from subacute endocarditis cases to retain the property for years after isolation is very striking. All these observations are based on inulin medium made up with peptone, according to Buerger,<sup>41</sup> as old methods are not reliable.

*Bile Test.*—When sufficient growth is obtained in neutral broth to perform a fair test of solubility in bile, this is the most reliable means of differentiating *Streptococcus mucosus* and pneumococcus on the one hand, from anhemolytic and hemolytic streptococci on the other. The 2 former are soluble, the two latter insoluble in bile. But the poor nutritive quality of the medium necessary for the performance of this test and the relative frequency with which insufficient growth for the test is obtained, makes it unsatisfactory. Whenever a good growth has been obtained the result always followed the rule stated. An organism, 4279, to be discussed in detail later (p. 438), was found on isolation to resemble a pneumococcus except for its insolubility in bile. On cultivation and animal passage it exhibited all the properties of an anhemolytic streptococcus.

*Blood Agar Plates.*—I have studied the appearances of the organisms in the original plain and glucose blood-agar plates, and when they



have been streaked out on the surface of plain ox-blood agar plates, as is seen in Table 2. In addition, I employed surface inoculations on heterologous human blood-agar plates and on 0.5% and 2% glucose ox-blood agar plates. From these studies the following statements may be made:

1. The presence of glucose in the medium diminishes and may entirely inhibit clearing.

2. The presence of glucose in the medium intensifies the production of green coloration. Organisms which do not produce green on sugar-free medium may produce a narrow greenish zone around the growth on glucose blood agar.

3. Clearing to be diagnostic of *Streptococcus hemolyticus* must appear within 24 hours and should consist of a zone 2-4 mm. wide.

4. Occasionally an organism which causes clearing in homologous blood (that is, medium made up with the blood of the patient from whom the organism is obtained) will not produce clearing on heterologous human blood.

A case of subacute streptococcus endocarditis with embolic gangrene of the leg yielded an organism which cleared in 24 hours in the original blood-culture plates, but which did not clear on heterologous human blood. While this phenomenon had been observed in a case of pneumococcemia and in a case of otitic sinus thrombosis (4319-4334, p. 440), it had never been observed in a case of this kind. These observations emphasize the necessity of employing blood other than that of the patient before making the final diagnosis of an organism. The cells of the patient may be so loaded with the toxic substances produced by the organism, that they break down very readily when additional poisons are formed by the growth in their immediate vicinity, whereas normal cells are not thus handicapped. It would be of interest to grow these organisms on agar made with the blood of another patient suffering from the same disease.

5. Occasionally an organism which clears on human-blood agar will not clear on ox-blood agar.

6. Organisms which produce a green coloration of the medium, when grown in the depth of a plate, frequently do not produce such coloration, when inoculated on the surface.

7. For diagnostic purposes the appearances on plain human-blood agar and sugar-free ox-blood agar are to be relied on. Surface inocu-

lations are more readily interpreted and more decisive than deep inoculations.

*Streptococcus mucosus* produces a mucoid, gelatinous, confluent, transparent growth, while the medium underlying it and for a considerable area about it becomes intensely green. The pneumococcus produces a moist grayish-green growth surrounded by a zone 2-4 mm. wide, of gradually deepening green medium, outside of which there is frequently a delicate line of clearing on ox-blood plates. Discrete surface colonies may show the ring form described by Buerger.<sup>12</sup> The ring colonies are best obtained by making an emulsion of pneumococci in a small amount of salt solution, and then pouring this over the surface of a glucose serum agar plate. The finding of ring colonies is often of great value in isolating pure cultures. Rarely a pneumococcus is obtained from the blood which clears in the original blood-culture plates, but does not clear in heterologous blood. Libman,<sup>39</sup> in 1905, isolated from the blood of a pneumonia patient a pneumococcus which produced concentric zones of clearing in the original plates. The organisms from the sputum of this patient grew typically green without clearing. Ruediger<sup>42</sup> reported in 1906 that he occasionally met with hemolytic pneumococci. None of the 24 pneumococci which I studied showed any of these variations.

The hemolytic streptococci produce a transparent colorless, a gray, or a white growth, the underlying medium and the surrounding medium for a distance of 2-4 mm. becoming perfectly free of blood pigment in 24 hours. Outside this clear area is a condensation or heaping up of blood pigment. Libman<sup>40</sup> recovered a streptococcus from the blood of a phlebitis patient which produced concentric zones of clearing, outside each of which was seen the condensation of pigment.

The anhemolytic streptococci produce a variety of appearances on blood mediums. Of the 10 streptococci from patients with subacute endocarditis, grown on human-blood agar, 3 produced white colonies, a green zone and linear clearing; 1, pale green growth, a green zone and linear clearing; 3, grayish growth, slightly green zone; 2, grayish white; and 1, gray, with no green pigmentation whatever. Of 9 of these organisms grown on sugar-free ox-blood agar one produced a grayish growth with underlying medium green; 1, transparent growth; 3, gray; 3, grayish white growth; 1, white. Thus 8 produced no green pigment whatever, in contradistinction to the constant produc-

tion of a well-defined green zone by pneumococci on sugar-free ox-blood agar. Of 4 anhemolytic streptococci from the blood of patients not endocarditic, grown on human-blood agar, 3 produced a dry gray growth, 1 an intensely green growth; while on ox-blood agar 2 produced a gray, and 1 a grayish-green growth. Of 8 anhemolytic streptococci from sources other than the blood stream, grown on human-blood agar, 1 grew gray and transparent, 1 white, 2 dry gray, 2 grayish-white, 1 grayish-white, with green pigmentation of the medium, and 1 intensely green. Of 7 of these grown on ox-blood agar, 2 grew gray and transparent, 1 white, 2 grayish-white, 1 grayish-green, and 1 pale green.

Thus we see that while a number of anhemolytic streptococci produced greenish pigmentation on human-blood agar, and a few on ox-blood agar, all the pneumococci produced a characteristic appearance on ox-blood agar plates, namely a grayish-green growth or deposit of organisms, a zone of gradually deepening green medium 2 to 4 mm. wide, limited by a delicate line of clearing. I wish to call attention to a characteristic appearance in the original plain-agar blood-culture plates of the cocci from patients with subacute endocarditis. There is the nucleus, pin-point to pin-head in size, formed by the white or gray opaque colony, surrounded by a demarcation zone of hazy partially cleared medium, outside of which is an areola of greenish pigmentation, or later on a narrow zone of clearing. The whole resembles the bull's eye of a target in appearance.

*Summary.*—In general, if an organism does not precipitate, it is either a pneumococcus or *Streptococcus mucosus*, and the capsule stain and the character of growth on glucose-serum agar and the blood agar determine which of these it is. If an organism precipitates it is almost surely a streptococcus, and the growth on blood agar determines whether it is hemolytic or anhemolytic. But every organism must be studied completely for some irregularity in its characters will reveal the variations taking place, especially among the anhemolytic streptococci.

#### ANHEMOLYTIC STREPTOCOCCI

Schottmüller,<sup>65</sup> in 1903, distinguished between streptococci which hemolyzed and those which did not hemolyze on blood agar. The latter he called *Streptococcus mitior* seu *viridans*. In blood plates, 2 parts of human blood to 5 parts of agar, deep colonies appeared as fine green points hardly pin-head in size. If only a few drops of

blood were added to the agar a narrow hemolytic zone appeared. Surface inoculations on blood agar yielded a fine gray or blackish-green deposit. Individual colonies were fine, almost colorless, later gray to greenish-black points. Some strains grew more richly on certain bloods and produced a narrow clear zone in the course of days. Schottmueller obtained such cocci from a great variety of local lesions of the mouth, nose, accessory cavities, and intestinal tract, and from the blood stream in a case of pancreatic necrosis, several cases of pyelephlebitis, and 7 cases of chronic streptococcus endocarditis. The endocarditis cases were clinically distinct, and he later called this disease "Endocarditis lenta." Much of his material was obtained post-mortem. His assertion that the organism is specific for endocarditis lenta can not be supported, for Horder<sup>32</sup> and Libman<sup>41</sup> have had cases caused by the influenza bacillus. While his bacteriologic studies lacked satisfactory capsule studies, the inulin, precipitation, and bile tests, he observed that surface cultures on blood agar did not produce as much green as deep colonies, that some strains slowly produced clearing, and that the concentration of blood was a factor.

Rosenow, in 1909 and 1910,<sup>54, 56</sup> described cases of chronic endocarditis caused by organisms which he called pneumococci. They differed from pneumococci of pneumonia cases in producing less green on blood plates, in losing the power to ferment inulin soon after isolation, in growing in clumps in fluid mediums, and in adhering closely to the surface of agar slants. This special character of clumpy and adherent growth was the more marked the more chronic the case, and the later in the course the organism was isolated; and was closely related to the power of producing endocarditis in animals. Artificial cultivation caused these characters to disappear after a variable time, all the strains gradually changing into typical lanceolate diplococci, often encapsulated, growing as typical pneumococci on blood agar and in broth. On animal inoculation the change occurred abruptly. "It has been impossible to so modify strains of *Streptococcus viridans* which these organisms much resemble." Agglutination studies seemed to show a closer relationship to ordinary pneumococci than to *Streptococcus pyogenes* and *Streptococcus viridans*. "The strains should be looked on as belonging to the pneumococcus group, and as having undergone environmental modification, and not to be regarded as strains of *Streptococcus viridans*."\* In 1910, Rosenow<sup>55</sup> stated that

\* Rosenow probably here referred to green-growing anhemolytic streptococci from sources other than endocarditis cases.



not all the strains fermented inulin, and that those which did ferment lost the property entirely after prolonged cultivation on blood agar.\* *Streptococcus pyogenes*, *viridans*, and *salivarius* did not ferment. Cocci from animals dying of endocarditis showed the original special characters, while those dying from bacteriemia or pneumonia soon after inoculation showed the characters of typical pneumococci. Reversion to normal type was caused by (1) prolonged cultivation, (2) short residence in normal serum, and (3) animal passage. Ordinary pneumococci grown in endocarditic serum acquired the special characters of endocarditic cocci. Endocarditis serums agglutinated normal pneumococci, but not hemolytic streptococci.

In his 1912 paper on experimental endocarditis, Rosenow<sup>37</sup> stated that while his organisms were identical with Schottmueller's and Horder's, they were to be regarded as pneumococci attenuated and modified by environmental conditions. Recognizing them as different from ordinary pneumococci, he considered them more closely related to typical pneumococci than to typical streptococci.

In 1910, Libman and Celler<sup>41</sup> showed that their cocci from cases of subacute endocarditis could be readily distinguished from hemolytic streptococci and pneumococci, when freshly isolated from the human body. They were small gram-positive, nonencapsulated cocci, all precipitating and resisting the lytic action of bile. One third fermented, two thirds did not ferment inulin.† Those that fermented retained the property over long periods of time. In original blood-culture plates the colonies were white, with or without a green or opaque zone about them. Subsequent cultures might show (1) a production of green pigment, (2) a moist white growth, or (3) a dry, almost colorless growth.

Libman and Celler said:

At first these organisms, especially those that do not ferment inulin, would appear far removed from pneumococci. But we have seen that pneumococci may lose their capsules, may lose the property of fermenting inulin, and may acquire the property of precipitation. Many of the organisms grow on blood plates like pneumococci. But we have not hitherto met with pneumococci which when isolated from the blood were resistant to the action of bile.‡ If we should meet with such pneumococci, we could more easily suspect that the organisms under discussion are altered pneumococci. The features of the endocarditis cocci have remained constant in subinoculations over a long period of time (in

\* This is contrary to our experience (p. 422).

† Of the 10 strains described in this paper, 7 fermented inulin. This large percentage may be accidental or due to an improved quality of inulin.

‡ Organism 4279 (p. 438) appears to be such.



some instances as long as eighteen months). Every organism that fermented inulin still does so. After repeated animal inoculations (mice, rabbits) no change in the important characteristics have been demonstrated. Capsules have never been developed.

At the International Congress in London, Libman<sup>43</sup> said that he had not been willing to state definitely that the organisms could come from pneumococci, although he believed they might, because he had

TABLE 3  
STREPTOCOCCUS ANHEMOLYTICUS FROM BLOOD OF CASES OF SUBACUTE ENDOCARDITIS

No.	Gram Stain and Morphology	Capsule	Glucose Broth	Precipitation
B	+ Round, ovoid, bacillary, lanceolate; pairs, short chains, clusters	Pneumococcus type	Flocculent growth; chains 4-8	+ Diminished after 7 transplantations
4110	+ Small, lanceolate and bacillary; short chains, diplococci	0	Discrete colonies; conglomerate convoluted chains	+
4147	+ Small, lanceolate and bacillary diplococci, clusters	0	Diffuse cloud; long convoluted chains	+
4170	+ Small, lanceolate and bacillary diplococci, clusters	0	Discrete colonies; conglomerate masses of chains	+
4206	+ Small, navicular, short chains and clusters	0	Diplococci, short, long and conglomerate chains	+
4275	+ Tiny, in chains and groups	0	Clumps; long convoluted and conglomerate chains	+
4026	+ Medium size diplococci, chains	0	Granular growth, convoluted chains	+
3975	+ Large cocci, diplococci, clusters	0	Diffuse cloud and clumps; diplococci, short chains	+
4342	+ Small, ovoid and lanceolate, chains	0	Diffuse cloud, chains of moderate length	+ 48 hr.
4357	+ Small lanceolate diplococci, short chains	0	Clumps, convoluted chains	+

not seen pneumococci that had become bile insoluble in the body, but having shortly before made such observation (Organism 4279 described in this paper), he believed the organisms in question could come from either pneumococci or streptococci, but that when isolated fresh from the body they were readily distinguished from both.

Eleven of 69 streptococci studied by Libman and Rosenthal<sup>42</sup> were anhemolytic. All were from local lesions. Seven grew white

and moist, 3 green, and 1 delicate and dry on blood agar. These organisms, it was stated, would correspond at least in part to *Streptococcus mitior* seu *viridans* of Schottnmueller. Up to July, 1912, no case of blood stream invasion by anhemolytic streptococci was recorded at the Mount Sinai Hospital, except in cases of subacute endocarditis.

English investigators, especially Andrewes and Horder,<sup>2</sup> found organisms corresponding in many respects to those which other writers

TABLE 3—*Continued*  
STREPTOCOCCUS ANHEMOLYTICUS FROM BLOOD OF CASES OF SUBACUTE ENDOCARDITIS

Inulin	Bile	Human-Blood Agar Plates	Ox-Blood Agar Plates
+	Not dissolved	Delicate dry growth; medium slight green tinge	
+	Not dissolved	Delicate grayish growth; narrow greenish zone	Grayish-white, linear clearing
+	Not dissolved	White colonies, green zone, linear clearing	Dry gray, linear clearing
+	Not dissolved	White colonies, green zone, linear clearing	Gray, linear clearing
+	Not dissolved	Gray growth	Transparent growth, linear clearing
+	Not dissolved	Grayish-white growth	Grayish growth, linear clearing
Slight fermentation in 48 hr.	Not dissolved	White colonies, green zone, linear clearing	Grayish-white, linear clearing
Reduction	Not dissolved	Pale green growth, green zone, linear clearing	Dry white growth, linear clearing
0	Not dissolved	Dry Grayish-white growth	Dry grayish-white growth
0	Insufficient growth	Grayish growth, underlying medium green	Grayish growth, underlying medium green

had isolated from cases of chronic infective endocarditis in the mouth, throat, and intestinal tract of man. They classified them, employing Gordon's tests, as *Streptococcus salivarius*, *anginosus*, and *fecalis*, but did not assert that the types were definite species. They stated that Schottnmueller's *Streptococcus mitior* corresponded to their *Streptococcus salivarius* and *fecalis*. In 24 cases of "malignant endocarditis" they isolated from the blood 2 *Streptococcus pyogenes*, 1 pneumococ-

cus, 6 *Streptococcus anginosus*, 4 *Streptococcus fecalis*, and 11 *Streptococcus salivarius*. "The first two organisms cause a rapidly fatal type, the others a chronic type which has nothing to do with rheumatism, except that this disease predisposes to infection of the valves by these low grade organisms." In a later paper, Horder<sup>32</sup> reported 88 cases of subacute and chronic bacterial endocarditis caused by *Streptococcus salivarius* and *fecalis*. Rarely *B. influenzae* or the pneumococcus was the cause. The pneumococcus cases may have been secondary terminal infections superimposed on and overwhelming the old infection.

TABLE 4  
STREPTOCOCCUS ANHEMOLYTICUS FROM BLOOD OF CASES OTHER THAN ENDOCARDITIS

Number	Disease Associated Organisms	Gram Stain and Morphology	Capsule	Glucose Broth
4049	Aseptic polynuclear plural effusion; 3 colonies, 72 hr.	+ Lanceolate, diplococci, short chains, clusters	Pneumococcus type	Diffuse cloud and flocculi; chains of 4-10
4343	Osteomyelitis of mandible.....	+ Small ovoid and lanceolate, clusters of diplococci	Narrow but indented (atypical)	Discrete colonies; chains of moderate length
4316	Postabortive infection with <i>B. coli</i> in blood and uterus	+ Small, round ovoid; diplococci, chains, clusters	0	Diffuse cloud short chains and small clumps
4348 B	<i>Streptococcus mucosus</i> meningitis; blood culture showed 1 colony <i>Streptococcus mucosus</i> , 1 colony of this organism	+ Small chains of diplococci	0	Granular growth; long, convoluted chains

Major,<sup>48</sup> in 1912, reported studies on 6 cases of endocarditis lenta, employing methods similar to ours. The organisms corresponded accurately with those studied by us, although none fermented inulin. He observed that surface inoculations on blood agar produced whitish colonies without green pigmentation, and that deep colonies produced a slight zone of hemolysis outside the green pigmented area. "We have never had sufficient evidence to consider these organisms as altered pneumococci." Tested according to the method of Andrews and Horder, they corresponded in part with *Streptococcus fecalis* and *Streptococcus salivarius*.

Recently Kinsella,<sup>34</sup> using the same methods, reported the same results in 12 cases. As 4 of the organisms grew indifferently on blood

agar, he preferred the name *Streptococcus mitis* to that of *Streptococcus viridans*. Only 2 strains fermented inulin, but the optimum medium for this test was not employed.

The classification based on fermentation reactions, of which Holman's is the most accurate, would seem to be more cumbersome than practical purposes warrant. Holman's studies show no relationship between the various types of anhemolytic streptococci and the pathologic conditions produced; for example, 4 types caused subacute endocarditis. Krumwiede and Valentine<sup>35</sup> concluded from agglutination studies that these streptococci constitute a heterogeneous group, and

TABLE 4—*Continued*  
STREPTOCOCCUS ANHEMOLYTICUS FROM BLOOD OF CASES OTHER THAN ENDOCARDITIS

Precipitation	Inulin	Bile	Human-Blood Agar Plates	Ox-Blood Agar Plates
+	+ Property lost on 4th transplant	Not dissolved	Dry grayish; medium unchanged	
+	0	Not dissolved	Dry gray	Dry gray
+	0	Not dissolved	Intense green	Grayish-green growth, linear clearing
+	0	Insufficient growth	Dry gray growth	Gray growth

there is no relationship between interagglutination and fermentation reactions. Henrici<sup>24</sup> could not corroborate the claims for specific organ affinity, nor for definite relationship between virulence and fermentation reactions.

In the course of one year's routine blood-culture examinations made during the course of a year, I isolated anhemolytic streptococci in 4 cases which presented no evidence of infective endocarditis. One case, 4343, was an osteomyelitis of the mandible, the infecting organisms coming no doubt from the mouth, where Horder finds *Streptococcus salivarius*. Another was a case, 4316, of postabortion infection, in which I obtained both from the uterus and the blood *B. coli* and *Streptococcus anhemolyticus*. Here the infecting organisms came undoubt-

edly from the intestinal tract, where Horder finds *Streptococcus fecalis*. In a 3rd case, 4049, an abacterial polynuclear-celled pleural effusion, 3 colonies of anhemolytic encapsulated streptococci were obtained from the blood. The 4th case, 4348 B, a meningitis due to *Streptococcus mucosus*, yielded 2 colonies in the blood-culture plates. One colony was a typical *Streptococcus mucosus*, the other was an anhemolytic streptococcus. I cannot say definitely whether this was a case of mixed infection (the meningeal infection was pure), or a case of mutation of the organism in the blood stream, but I am inclined to favor the latter view.

The finding of such organisms in the blood of patients not suffering from subacute endocarditis was a new experience at the hospital laboratory. It might be feared that this would lessen the diagnostic value of the blood culture in subacute infective endocarditis, but the function of the laboratory is to confirm a diagnosis suspected from the clinical symptoms. Where other sources of bacteriemia can be excluded and where sufficient clinical phenomena are present, the finding of anhemolytic streptococci in the blood still indicates the existence of an infection of the endocardium.

The cultural characters of the 22 anhemolytic streptococci from local lesions, from the blood in cases not endocarditic, and from the blood in cases of subacute endocarditis are detailed in Tables 3, 4, and 5. The following conclusions may be drawn from these data:

1. "*Streptococcus viridans*" is a confusing name for the organisms causing subacute endocarditis, and should be abandoned. A considerable number of the strains produce no green whatever on blood-agar plates. The organism is not specific for the disease; it may cause other lesions, and other organisms, notably the influenza bacillus, may cause the lesions of the disease.

2. These organisms occur as saprophytes in the mouth, throat, and intestine of man. They may acquire parasitic properties, produce local lesions, or invade the blood with or without infecting the endocardium. They are seldom found in the blood stream, however, except in the cases of subacute streptococcus endocarditis.

3. While the anhemolytic streptococci from the cases of endocarditis exhibit much more uniformity and fixation of cultural characters than those from other sources the limits of variation are so wide that accurate cultural differentiation is impossible. The organisms from endocarditis patients are very rarely encapsulated. A considerable



number ferment inulin rapidly and intensely, and retain that power over long periods of time (surely for 5 years) when artificially cultivated.\* The anhemolytic streptococci which ferment inulin but come from other sources do not retain the property so tenaciously.

Complement-fixation studies by Dr. Olitsky with the serums of patients suffering from subacute streptococcus endocarditis have given remarkable results. With antigen prepared from the homologous organism, there is a marked fixation; with the mixed antigens made from a number of organisms isolated from the blood of other endocarditis patients, there is a positive but less marked fixation. There is no fixation with a mixed antigen made from strains of similar anhemolytic streptococci isolated from the throat and local lesion. These facts indicate that anhemolytic streptococci from cases of endocarditis assume certain biologic properties after they have invaded the blood stream and infected the endocardium. This corresponds with the greater uniformity of cultural characteristics which we found among them than among strains of anhemolytic streptococci from local lesions. The relatively long residence of the cocci in the body in cases of endocarditis undoubtedly is an important factor in the development of its more uniform cultural and biologic characteristics.

4. There is no correlation between the type of growth in blood medium, the production of green, etc., and the pathologic condition produced. Some from endocarditis patients are gray, white, or colorless, while some from local lesions produce intense green pigmentation.

5. The organisms may be roughly divided into 4 groups, thus:

- (a) Capsules present: Inulin +
- (b) Capsules present: Inulin ○
- (c) Capsules absent: Inulin +
- (d) Capsules absent: Inulin ○

But here again no constant correlation with the disease produced can be discerned. The cocci from subacute endocarditis belong, with a few exceptions, to Groups c and d.

6. The common properties of anhemolytic streptococci are these: They are chain-growing cocci, gram-positive, insoluble in bile, precipitate, and do not cause clearing in 24 hours when streaked on plain blood-agar plates.

In view of these facts I believe that the various names given to these organisms by authors should be discarded for the present, and

\* This is in striking contrast to the observations of Rosenow (p. 426).

that we should speak of them simply as *Streptococcus anhemolyticus*. In comparative studies of *Streptococcus anhemolyticus* isolated from cases of subacute endocarditis and of *Streptococcus anhemolyticus* found in connection with some cases of polyarthrititis, we may speak of the former as endocarditis streptococci (anhemolytic), and of the latter as arthritis streptococci (anhemolytic).

#### VARIATION AND MUTATION

Ruediger,<sup>64</sup> in 1906, reported that 2 hemolytic streptococci grown in glucose broth for 2 years had lost the property of clearing blood agar. Buerger and Ryttenberg,<sup>16</sup> in 1907, observed changes occurring in pneumococci in the human body. From the blood of a patient with puerperal infection, they obtained an encapsulated hemolytic organism which 'reverted' to a pneumococcus when passed through white mice. An abscess of the eye developing in this case yielded an organism with pneumococcus capsule and streptococcus cultural characters which could not be reverted. An atypical organism was isolated from an acute osteomyelitis; a 2nd operation some time later yielded typical pneumococci. Here, reversion had taken place in the body. They found that pneumococci may acquire streptococcus properties in the human blood or exudate; that such organisms may revert in the body or in animal passage; that the acquired properties may become fixed by metastasis or by cultivation on mediums; that not all atypical strains can be reverted, and that not all the organisms from 1 case may permit of this change. The cultural studies lacked systematic blood-plate observations, and the bile test was not yet in use. The authors relied on the type capsule as the criterion for differential diagnosis, but said that others might look on their original organisms as atypical streptococci. In the absence of the bile test and accurate blood plate studies, no definite opinion may be formed, but I am inclined to look on 2 of their organisms as hemolytic and anhemolytic streptococci (Case 1 and Case 2) which they succeeded in changing into pneumococci.

Rosenow,<sup>58</sup> in 1912, reported that cultivation on blood agar changed the streptococci of epidemic sore throat into hemolytic streptococci, but that 1 soaking in sterile unheated milk reverted them. A strain grown for 6 months on blood agar reverted to original type on passage through 2 guinea-pigs. A strain grown in milk and passed through a guinea-pig resulted in growth resembling *S. mucosus*. Hemolytic streptococci from scarlet fever when grown in unheated sterile milk could be made to acquire capsules and to lose much of their hemolytic property. He

also noted at this time that drying of the medium with salt concentration made the epidemic organisms hemolytic.

Davis,<sup>20</sup> in 1913, converted a hemolytic streptococcus into a mucosus by passage through a guinea-pig. Intravenous injection of a rabbit with *St. mucosus* produced suppurative arthritis, from which a hemolytic streptococcus was obtained.

Rosenow<sup>60</sup> in his paper on "Transmutations within the Streptococcus-Pneumococcus Group" reviewed the literature on this phase of the subject, and presented his own experiments. He asserted that *Streptococcus hemolyticus*, *viridans*, *rheumaticus*, the *pneumococcus*, and *Streptococcus mucosus* are so closely related that one may change into the other under proper conditions. His methods consisted of prolonged cultivation on blood agar, drying of the medium, varying the salt concentration of the medium, use of oxygen pressure, symbiosis with *B. subtilis*, and animal passage. Not only did he find that the organisms change culturally, but also that their characteristic serologic reactions and their characteristic "elective affinity" for definite structures in experimental animals are exhibited in their new guise. The organisms are changed not only morphologically and culturally, but biologically as well. These studies have apparently demonstrated relationships which various authors have suspected from isolated observations.

I had the opportunity to study with Dr. Libman a number of organisms submitted by Dr. Rosenow. Some of these represented strains before and after mutation had been effected. Our cultural studies led us to conclude that Dr. Rosenow's criteria for diagnosis were the same as our own.

Two criticisms of the mutation studies have been made. The 1st has been made by upholders of the "Pure Line Concept." Cole and Wright<sup>18</sup> point out that since bacteria are asexual and do not propagate by amphimixis, a pure line consists of the descendants of any single cell, constituting a clone. Within species are cultural races or varieties, each with its own characteristics and range of variability, which exist side by side independent of environment. Each strain transmits the impress of its recent history.\*

According to the exponents of the theory mutations previously reported can be accounted for by the presence of 2 or more strains in

\*Observed differences due to slight variation of medium are not true variations. True protoplasmic variations under identical conditions are of 2 kinds: (a) due to causes operating within the cell; for example, unequal division of nuclear material. These are usually minute "fluctuating variations," rarely "mutations or sports;" (b) impressed variations (remembering the tremendous number of generations produced by bacteria in a short time).

the original culture. Under varying conditions, one or the other strain predominates in subcultures. If the conditions are maintained for a short time only, 1 strain is suppressed and when original conditions are restored, this strain reappears, gains predominance, and produces an apparent reversion. If, however, the adverse environment persists for a longer time, the less favored strain dies off and cannot reappear

TABLE 5  
STREPTOCOCCUS ANHEMOLYTICUS FROM SOURCES OTHER THAN THE BLOOD STREAM

Number	Source	Gram Stain and Morphology	Capsule	Glucose Broth
24862	Sputum; lung abscess	+ Small short chains	Close fitting atypical	Flocculent growth; chains of 4-12
S 41	Excised tonsil	+ Small lanceolate diplococci, short chains, groups	Pneumococcus type; lost on transplantation	Granular growth; long convoluted conglomerate chains
26911	Pelvic abscess	+ Moderate size lanceolate and navicular, groups	Narrow, indented, atypical	Diffuse cloud; diplococci; chains of 8-12
C	Sore throat	+ Small navicular, diplococci, clusters	0	Granular growth; long convoluted chains
26941	Bile, acute cholecystitis	+ Large, round, irregular, grouped	0	Granular growth; long chains
26496	Sputum	+ Small diplococci in clusters	0	Granular growth; long and short chains, clumps
26891	Bile; carcinoma of liver and pancreas	+ Moderate size lanceolate and navicular; irregularly grouped	0	Granular growth; short chains and clumps
26942	Urine	+ Lanceolate	0	Clumpy growth; chains of moderate length

when original conditions are restored, thus producing an apparent permanent mutation. This contention casts doubt on our current bacteriologic methods of obtaining pure cultures, and would seem to necessitate the use of Barber's method for the cultivation of single organisms.

Holman's experiments<sup>28</sup> on symbiosis of streptococci with other varieties, pneumococci, and other bacteria lend support to these contentions. He points out that plating from blood agar to blood agar

without preliminary growth in serum broth is very apt to give impure colonies.

The 2nd objection has been raised by Holman, who has demonstrated that animal passage as a means of producing mutations is a very fallible method. Spontaneous infection with various types of streptococci and with pneumococci occurred in guinea-pigs untreated

TABLE 5—*Continued*  
STREPTOCOCCUS ANHEMOLYTICUS FROM SOURCES OTHER THAN THE BLOOD STREAM

Precipitation	Inulin	Bile	Human-Blood Agar Plates	Ox-Blood Agar Plates
+	+	Not dissolved	Dry gray	
+	Slight, 0 2 months later	Insufficient growth	Delicate, gray transparent growth	Delicate, gray transparent growth
+	0	Not dissolved	Dry gray	Grayish-white
+ 48 hr.	+	Not dissolved	Intense green	Pale green growth, green zone, linear clearing
+	+ 48 hr.; lost on 3rd transplant; property recovered by passing through mouse	Insufficient growth	Grayish-white	Grayish-white
0 at first + on 3rd transplant and mouse injection	0	Not dissolved	Grayish-white growth, medium green	Grayish-green growth
+	Reduced	Not dissolved	White	White
+	0	Insufficient growth	Grayish-white	Grayish, transparent

or injected with other bacteria. He believes that many of Rosenow's transmutations in animals were secondary invasions from the animals themselves.

Among the 71 organisms studied by me, many minute variations due to changes in the medium were observed, but they were temporary in nature. Some permanent variations occurred, such as loss of capsule, loss of power to ferment inulin, loss or acquisition of precipitation, etc., but these changes were not sufficient to change the classification



of the organisms according to my standards. Two definite mutations were encountered and the detailed observations follow:

Organism 4279 was isolated from the blood\* in a case of brain abscess and sinus thrombosis following otitis media, on March 30, 1913. Its characters were as follows:

A gram-positive coccus was seen in pairs and chains; lanceolate, navicular, and bacillary forms.

The colony from original blood-culture plate showed only suggestion of a capsule, but the transplant on fresh 2% glucose-serum agar showed a well defined, indented capsule, approaching the pneumococcus type.

Glucose broth gave a clumpy growth; conglomerate masses of chains.

There was no precipitation.

Inulin fermented in 48 hours.

It was not dissolved in bile.

Human-blood plates gave delicate pin-point grayish-white colonies, with wide green zone and linear clearing. Ox-blood, grayish-green growth, the medium unchanged.

It was difficult to classify this organism for its morphology, capsule, fermentation of inulin, lack of precipitation and green growth spoke for the diagnosis of pneumococcus, while the atypical growth on ox-blood agar, the clumpy growth in glucose broth, and its insolubility in bile allied it to the anhemolytic streptococci. If the organism had been soluble in bile, I should have considered it a slightly atypical pneumococcus. Was I perchance dealing with an organism in the very process of changing from a pneumococcus into a streptococcus, the first evidence of which change was its insolubility in bile?

On its 3rd transplantation (at 4- or 5-day intervals), on 2% glucose-serum agar, precipitation of the medium appeared.

April 16, 1913. The growth from 1 slant was injected into the peritoneal cavity of a mouse, and the animal killed 24 hours later. Culture of the slight exudate gave an organism with a wider, more typical pneumococcus capsule, causing precipitation not only of 2% glucose-serum agar, but also about the condensation water of 0.5% glucose-serum agar. Otherwise it remained unchanged.

April 24, 1913. The culture from the 1st mouse was injected into a 2nd mouse. The organism obtained showed a wide typical pneumococcus capsule, precipitated on 0.5% glucose-serum agar, but did not ferment inulin. Otherwise it remained unchanged.

The organism now corresponded in every way to a green-growing anhemolytic streptococcus, possessing a pneumococcus-type capsule, insoluble in bile and not fermenting inulin.

May 3, 1913, the original organism and the 2 strains from the mice were all found not to ferment inulin.

May 18, 1913, the 3 cultures were studied completely and were now found to have no capsule, precipitated, did not ferment inulin, were not soluble in

\* It is of importance to note that only 1 colony in 1 blood plate was obtained. This excludes the possibility of 2 strains being present from the outset.

bile, and grew grayish-green on ox-blood agar. They had lost their capsules and their power of fermenting inulin, and, therefore, corresponded with the anhemolytic, nonencapsulated streptococci.

The view might be taken by some that the organism was an anhemolytic streptococcus which had acquired certain pneumococcus characters in the human body, and which on cultivation and animal passage had reverted to its original type. But I regard this organism as a pneumococcus which, when isolated from the body, had already begun to change into a streptococcus, as evidenced by its insolubility in bile. It would seem that this change in reaction to the bile test is the first to occur when mutation takes place and is, therefore, of biologic importance. Then in their turn came the acquirement of the property of precipitation, the loss of power to ferment inulin, and finally the loss of the capsule. The latter was maintained for some time because of animal passage, but soon disappeared on artificial cultivation.

Two points deserve emphasis: (1) I was dealing with a pure line; (2) the final change was the same with the original strain kept on culture mediums as with 2 strains after passage through 2 different mice. These observations answer the 2 objections which have been made against mutation.

Organism 4319 was isolated from the blood in a case of otitic sinus thrombosis, on April 30, 1913. Its characters were as follows:

A very small gram-positive coccus was seen in pairs, short chains and groups like staphylococci. No capsule enveloped the organism.

Glucose broth gave sparse growth. Short chains and large clumps were observed.

Precipitation was present.

Inulin was not fermented.

It was not dissolved in bile.

Original blood-culture plates showed small grayish-white colonies surrounded by a wide zone of clearing.

Streak cultures were intensely green on plain blood agar, made up with another human blood.

There was a grayish-green growth with only linear clearing on ox-blood agar.

This organism was classified as an anhemolytic streptococcus which had acquired the property of hemolyzing on the blood of the host.

On May 13, 1913, a 2nd blood culture, 4334, was taken because, in spite of operation, arthritic symptoms and cutaneous manifestations of acute 'malignant' endocarditis had appeared. The organism now showed the same morphology, no capsule, precipitated, did not ferment inulin, and was insoluble in bile. In glucose broth, it produced a granular growth with long convoluted chains. On blood-agar plates, it not only hemolyzed the host's blood, but heterologous human blood and ox blood as well.

This streptococcus, originally anhemolytic, acquired first the power of hemolyzing the host's blood, and then as it became engrafted on the endocardium, it acquired the property of hemolyzing other blood as well.

Organism 4348 B. A blood culture was made in a case of streptococcus mucosus meningitis. Two colonies were obtained, 1 of which was a typical *S. mucosus*. The 2nd was a nonencapsulated gray-growing streptococcus (Table 4).

The culture was taken a few days before death, and this organism cannot be regarded as an antemortem invader. I am inclined to regard it as a mutation taking place in the body. Injection of mice with cultures gave no results.

In our experience we have observed, therefore, the following mutations:

1. Encapsulated hemolytic streptococcus converted into a pneumococcus (Buerger and Ryttenberg, Case 1).

2. Encapsulated anhemolytic streptococcus converted into a pneumococcus (Buerger and Ryttenberg, Case 2).

3. Pneumococcus changed into anhemolytic streptococcus (Organism 4279).

4. Anhemolytic streptococcus converted into a hemolytic streptococcus (Organism 4319-4334).

5. Streptococcus mucosus changed into an anhemolytic streptococcus (Organism 4348 B).

We believe that mutations occur in nature, that they occur uncommonly, and that their occurrence is not of sufficient frequency to interfere with current bacteriologic methods, or with the practical application of those methods to clinical medicine.

Smith and Brown<sup>68</sup> say: "Spontaneous changes in cultural characters do not proceed rapidly enough, if they go on at all, to interfere with current bacteriologic methods."

#### THE RHEUMATIC COCCI

The literature of investigations into the etiology of rheumatism (acute rheumatic fever, acute polyarthritides) may be found in the writings to be mentioned, especially in "Researches on Rheumatism," by Poynton and Paine.<sup>52</sup> I wish merely to present brief descriptions of the better known organisms believed to be the etiologic factors of the disease.

The diplococcus rheumaticus of Poynton and Paine is described by the authors as follows:

Minute cocci in pairs, averaging 0.5 micron in diameter.

Stains readily with anilin dyes but does not retain Gram's stain with great tenacity. Stains best in tissues with carbol-thionin.

As a rule, it shows no capsule, but in human tissues an appearance of capsulation may be noticed occasionally.

In liquid media they grow in chains of varying length. In solid media they grow in masses that resemble the arrangement of staphylococci.

They grow aerobically but better anaerobically. Best medium consists of milk and broth acidified by lactic acid.

On blood agar minute white colonies appear in 24 hours. These tend to remain discrete and to alter the blood pigment to a rusty brown color.

Walker and Ryffel<sup>76</sup> and Beaton and Walker<sup>5</sup> found that similar organisms which they isolated in cases of rheumatism grew well in alkaline medium, as described by Wassermann, and produced large amounts of acid, chiefly formic. They also first described the rusty brown or chocolate color produced on blood agar, which they attributed to the formation, by reduction, of hemochromogen. Beattie<sup>6</sup> obtained a micrococcus from 2 joints, but reported no studies on their cultural characters.

Shaw,<sup>67</sup> studying cultures from Wassermann, Poynton and Paine, and Walker concluded that the 3 organisms were identical, and the cause of acute rheumatism.

Andrewes and Horder<sup>2</sup> found Poynton and Paine's diplococcus rheumaticus to be a streptococcus salivarius, and Beattie's diplococcus to be a streptococcus fecalis when subjected to Gordon's tests.

Rosenow,<sup>55</sup> in 1910, stated that *Micrococcus rheumaticus* might or might not ferment inulin, and grew green on blood plates. Major,<sup>48</sup> in 1912, stated that his cultural studies of Poynton and Paine's No. 34, Beattie's 254, and Lintz's 399 showed no essential differences between them and the organism of 'Endocarditis lenta.'

Various views are held as to the relationship of rheumatism to "malignant endocarditis." Thus Poynton and Paine believe that rheumatism may cause both simple and malignant endocarditis. In simple rheumatism the blood rarely yields the diplococcus because the organisms are localized in closed cavities, such as synovial, pericardial, etc., and not being on the surface of the heart valves are destroyed by phagocytosis and sclerosis. In the necrotic material constituting the vegetations of simple rheumatic endocarditis, the infection lurks quiescent, and is as dangerous as a necrotic tuberculous gland bordering

on the blood stream. In 'malignant rheumatic endocarditis,' the organisms reach the surface of the valve, multiply rapidly, and are discharged into the blood stream. Poynton and Paine find that the organisms from these 2 types of rheumatic endocarditis are so similar as to be considered identical, that the organism from 1 type can produce both types in animals, that these 2 types are forms of 1 and the same disease.

By most authors this conception is not held, the great majority describing as a separate clinical entity the disease known as subacute and chronic infective or malignant endocarditis or endocarditis lenta. The malignant rheumatic endocarditis of Poynton and Paine is undoubtedly the same disease.

Most authors agree with Andrewes and Horder that rheumatism is related to subacute endocarditis only in so far that by damaging the valves it predisposes them to infection by low grade streptococci. They believe that the organisms of Poynton and Paine are agonal invaders or terminal infecting agents, or come from cases of endocarditis lenta. Horder, in 1906, cast much doubt on the validity of Poynton and Paine's assertions. His cultures of the blood in rheumatism were uniformly negative; in chronic "malignant endocarditis," almost always positive. Cultures from rheumatic tonsils and tissue and exudates yielded discordant results. Much doubt is cast on the importance of animal experiments, for saprophytic streptococci from throat and intestine produced arthritis and endocarditis. How is it, he asks, that if simple rheumatism so rarely yields positive blood cultures, for reasons given by Poynton and Paine, that Beaton and Walker obtained 3 positive blood cultures by merely pricking the ear? If the blood contains so many organisms, why do many reliable investigators fail to grow the organisms?

In reply to the criticisms of their work Poynton and Paine offer the fact that patients from whom they isolated the diplococcus have gone on to complete recovery and lived for years afterward, and that, therefore, the organisms cannot be regarded as agonal invaders.

Much support apparently is given to the contentions of Poynton and Paine by the recent writings of Rosenow on the transmutation of organisms<sup>60</sup> and on the etiology of rheumatism.<sup>59</sup> Using semi-anaerobic methods he cultivated organisms from the joints of 14 of 16 cases. From the blood, using the usual methods, he obtained 1 positive result in 3 cases, but by laking the blood before inoculating the mediums he



obtained 3 positive cultures of 4 attempts, that is, a total of 4 positive blood cultures in 7 cases. The organisms fall into 3 groups as follows:

1. Five strains. Long chains of diplococci, large clumps in broth, green on blood agar.

2. Six strains. Size of hemolytic streptococci, short chains and diplococci, slight hazy hemolysis on blood agar. This group especially found in the type of rheumatism with muscular manifestations.

3. Three strains. Clumps of micrococci, occasionally short chains and diplococci, small grayish colonies on blood agar.

None ferments inulin, all produce large amounts of acid. In order of virulence come Groups 2, 1, 3 (least). Rosenow has converted one type into the other, and all into typical pneumococci. Organisms of Group 1 on prolonged cultivation come to resemble *Streptococcus viridans*. In addition, he has converted 3 pneumococci into rheumatic streptococci. Rosenow believes that the organisms of the pneumococcus-streptococcus group may undergo mutation within the body and thus produce different specific pathologic lesions in the body at different times. If this is true, much support is given to the theory of Poynton and Paine.

Up to the present we have not been able to confirm the assertions of Rosenow as to "elective affinity," although we have no doubt that it holds at least to a certain extent (and clinical experience alone would make us give some credence to the view).

In this connection the studies of Rothschild and Thalhimer<sup>73</sup> are of importance. These authors employed for their experimental studies the same organisms described in the present paper. They employed the name *Streptococcus mitis*, however, for the anhemolytic streptococcus of subacute endocarditis. They produced arthritis in 50% of rabbits injected with endocarditis streptococci and endocarditis in only 7%, results which are the reverse of Rosenow's. This experimental arthritis differed in no way from that reported by investigators of the *Streptococcus rheumaticus*, nor from the form of arthritis produced by the authors in 45% of rabbits injected with 5 strains of this organism isolated by Poynton and Paine, Beattie, Lintz, and Rosenow. They concluded that "the deduction of a distinct variety or species of streptococcus based upon the power to cause arthritis in rabbits is unwarranted." The studies of Krumwiede and Valentine<sup>35</sup> and of Henrici<sup>24</sup> corroborate the lack of specificity indicated by their studies.

Swift and Kinsella<sup>70</sup> took 85 blood cultures in 58 cases of rheumatism, and obtained 7 positive cultures in 6 cases. Repetition a short

time later was negative in all but 1 patient. The bacteriemia was, therefore, fleeting, in sharp contrast to the persistence of bacteriemia in subacute streptococcus endocarditis. The organisms were anhemolytic streptococci, nonencapsulated, insoluble in bile, 2 strains fermenting inulin. The fermentation tests showed no uniformity, but optimum medium was not used for these tests. Joint cultures made in 34 cases proved sterile.

TABLE 6  
STREPTOCOCCI FROM CASES OF RHEUMATISM, ISOLATED BY VARIOUS AUTHORS

	Gram Stain and Morphology	Capsule	Glucose Broth
Poynton and Paine 34 From Mus. Nat. Hist.	+	Pneumococcus type on many	Diplococci; long and short straight chains
	Large; lanceolate diplococci in chains		
Poynton and Paine 34 From Dr. Rosenow	+	0	Long chains
	Large; lanceolate diplococci in chains		
Beattie 254 From Mus. Nat. Hist.	-	0	Granular growth; diplococci; short chains; groups
	Ovoid coccus; pairs and groups		
Beattie 254 From Dr. Rosenow	+	Some 0; mostly with pneumococcus type	Diffuse clouding; diplococci; short chains
	Ovoid coccus; pairs and groups		
Lintz 339 From Mus. Nat. Hist.	+	Pneumococcus type	Granular growth; diplococci; short chains; groups
	Lanceolate diplococcus; short chains and groups		
Lintz 339 From Dr. Rosenow	+	0	Granular and mucoid growth; very long chains
	Large, lanceolate diplococci in chains		
Rosenow 735 April, 1913	+	0	Granular growth; conglomerate masses of chains
	Lanceolate singles; diplococci; short chains; groups		
May, 1913	Unchanged	except	
Rosenow 738a From tonsil April, 1913	+	Narrow, poorly staining; atypical	Sparse, clumpy growth; long chains; some conglomerate
	Lanceolate, singles; mostly diplococci; short chains and groups		

I have had for study 3 rheumatic cocci obtained from the Museum of Natural History, and the same strains from Dr. Rosenow. These have been studied in the usual manner and the results recorded in Table 6. It is interesting to note that the same organisms from different sources show differences in their characters. This may be due to differences in the nutritive medium on which they have been grown, climate, etc. Thus the museum cultures were kept on serum agar. Rosenow's on blood agar.

The organisms fall into the class of anhemolytic streptococci. They are gram-positive, may possess a typical pneumococcus capsule, form large amounts of acid, as shown by the rapid and intense precipitation, do not ferment inulin, are not dissolved by bile, and on blood plates grow grayish-white with no production of green and no clearing, with the exception of 1 organism (Poynton and Paine 34, Museum of

TABLE 6—Continued  
STREPTOCOCCI FROM CASES OF RHEUMATISM, ISOLATED BY VARIOUS AUTHORS

Precipitation	Inulin	Bile Test	Human-Blood Agar	Plain Ox-Blood Agar
+++	0	Not dissolved	Grayish-white growth; moderate clearing	Grayish-white; linear clearing
+++	0	Not dissolved	Grayish-white; no clearing	Grayish-white; linear clearing
+++	0	Not dissolved	Grayish-white; no clearing	Grayish-white; linear clearing
+++	0	Insufficient growth	Grayish-white; no clearing	Grayish-white; linear clearing
+++	0	Not dissolved	Grayish-white; no clearing	Grayish-white; linear clearing
+++	0	Insufficient growth	Grayish-white; no clearing	Grayish-white; linear clearing
+++	0	Not dissolved	Clearing	Delicate transparent growth; no clearing
				Grayish-white growth; underlying medium cleared
0 Except occasionally about the condensation water	0	Not dissolved	Dry grayish-white growth	Grayish-green growth; underlying medium green

Natural History), which cleared on human blood agar but not on ox-blood agar. The rusty brown or chocolate color on blood agar noted by English observers has not been seen on plain blood-agar plates, but on glucose blood-agar plates the organisms produce in 48 hours a brownish growth, surrounded by a wide zone of hazy medium, which is partly cleared but precipitated as well. This appearance has not been observed with any other organisms, and is probably due to the intense production of acid from the glucose of the medium.

The organisms are thus seen to differ slightly from the streptococcus of subacute endocarditis. The pneumococcus-type capsule is more frequent in the former and is apparently retained for a long time on artificial medium. They produce large amounts of acid, as evidenced by their intense power of precipitation and by the peculiar growth on glucose blood agar.

Employing our present blood-culture technic and the Rosenow technic, we have never obtained positive results in cases of acute articular rheumatism. Hence the problem of differentiating such organisms from those we have so often isolated in cases of subacute endocarditis has not arisen. If we are confronted by such a problem, I believe that the clinical course and symptoms will have to be our guide in the differential diagnosis. Moreover, the bacteriemia thus far found in cases of rheumatism, is fleeting, while that of subacute endocarditis is very persistent. Positive results in the former apparently require special methods, while in the latter the routine procedure yields a high percentage of positive culture.\*

I have also had for study 2 of the organisms isolated by Rosenow, 735 and 738a. Of these, the 1st seemed to be a hemolytic streptococcus, and the 2nd an anhemolytic encapsulated streptococcus which lacked the property of precipitation, that is, an intermediate organism. At any rate neither conformed culturally with the cocci of the other authors.

It must be admitted that no fair conclusions can be drawn from cultural studies made on organisms isolated a long time ago and kept alive on various artificial mediums for long periods of time. We know that such conditions may produce marked changes in cultural and morphologic characteristics, and pathogenic properties as well.

As regards the true significance of anhemolytic streptococci found in the blood of cases clinically considered as instances of rheumatic fever, a final opinion cannot be given at present. Some of these cases are really instances of metastatic arthritis secondary to a definite primary inflammatory or purulent focus. Such cases should be named according to the organism isolated from the primary focus, the joints, or the blood. In clinical medicine cases of arthritis secondary to a primary focus due to streptococcus hemolyticus are now properly termed "streptococcus (hemolyticus) arthritis." It will be important

\* Further studies may reveal more definite characteristics for the arthritis streptococci (anhemolytic) than those pointed out in the 2 preceding paragraphs.

to determine whether the streptococci found in connection with true rheumatic polyarthritis are secondary invaders, a possibility entertained by Swift and Kinsella.

#### SUMMARY

For the differentiation of the organisms of the pneumococcus-streptococcus group for clinical diagnostic purposes, a morphologic and cultural method which has yielded good results has been presented. Based on a study of many organisms according to this method, a classification has been adopted which seems to be as definite as can be attained in our present state of knowledge. There is much evidence that the classification is artificial from a biologic point of view, as shown by the variations within the classes and by the existence of intermediate and transitional forms, as well as by the mutations which have been described.

The limitations of the method are seen in the study of the anhemolytic streptococci. In this class are included bacteria leading a saprophytic existence in the mouth, throat, and intestinal tract of man, bacteria producing local infections in adjoining parts, bacteria producing a bacteremia, notably when the endocardium is the site of the local lesion, bacteria thought to be the cause of ordinary acute articular and muscular rheumatism, and of simple rheumatic or verrucose endocarditis. The limits of variation within this class of organisms are so wide that their separation into definite types associated with definite pathologic lesions does not at present seem possible by this method of study. According to our own experience, the finding of anhemolytic streptococci in the blood stream usually indicates an infection of the endocardium. The organisms from these patients exhibit certain peculiarities which are more or less distinctive, but in every case the laboratory finding serves as in the case of other bacteremias, to confirm a diagnosis justified by the clinical symptoms. I would suggest that for practical purposes the anhemolytic streptococci from cases of subacute endocarditis be called endocarditis streptococci (the name *viridans* being misleading), while those organisms isolated from cases of polyarthritis be called arthritis streptococci.

With regard to the bacteriologic method itself, I wish to emphasize certain points. All studies to be of value should be made on organisms freshly isolated from the body for there is sufficient evidence to show that not infrequently they undergo changes when cultivated. Studies should be complete so that more evidence of the existence of inter-



mediate and transitional forms may be established. For the inulin test the Buerger modification of the Hiss medium should be used. Blood-plate studies are especially instructive and should include not only the observation of the original blood-culture plates, but also streak cultures on heterologous human and ox-blood agar plates. It is essential that the agar should be free of glucose in blood-plate studies.

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## THE ETIOLOGY OF COMMON COLDS \*

### THE PROBABLE ROLE OF A FILTRABLE VIRUS AS THE CAUSATIVE FACTOR: WITH EXPERIMENTS ON THE CULTIVATION OF A MINUTE MICRO-ORGANISM FROM THE NASAL SECRETION FILTRATES

#### PLATE 9

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Numerous attempts have been made to demonstrate the specific cause of the extremely prevalent catarrhal affections of the upper respiratory tract, popularly known as "common colds." In reviewing the literature, however, one is struck by the multiplicity of organisms to which an etiologic relation has been ascribed. *B. influenzae*, *Strep. hemolyticus*, *Strep. viridans*, the pneumococcus, *M. catarrhalis*, *B. septus*, Friedlander's bacillus and Tunick's *B. rhinitis* have each held the stage. Some investigators believe that micro-organisms bear no relation whatever to the causation of colds; but that many factors—wet and cold, drafts, irritating vapors, overheated rooms, worry, fatigue, sexual excesses, dietetic errors, alcohol, and what not—enter into the etiology. Still another group takes the stand that a number of conditions—notably chilling, sudden changes in temperature, and fatigue—act as predisposing factors by lowering resistance, and thus pave the way for infection by micro-organisms commonly believed to be present normally in the nasal cavities.

In short, present knowledge of the etiology of the common cold is in a most chaotic state. As remarked by Stitt,<sup>1</sup> "Of all the diseases common in man this condition has been surrounded by greater etiological and epidemiological obscurity than any other."

#### CLINICAL DEFINITION OF THE "COMMON COLD"

Much confusion in the interpretation of the reported findings of various investigators has resulted from classifying various respiratory infections under one heading. It is an almost universal custom to

\* Received for publication June 18, 1917.

<sup>1</sup> Practical Bacteriology, Ed. 4, 1916, p. 435.

classify as common colds mild and transitory rhinorrheas, without systemic manifestations and due to vasomotor disturbances; the ordinary acute coryza, with its slight or moderate systemic disturbances; and the so-called grip or influenza, characterized by more or less marked systemic reaction and prostration.

The cases studied in this investigation conform to the entity designated by Osler<sup>2</sup> "acute catarrhal fever (acute coryza)," the symptoms of which he describes as follows:

The patient feels indisposed, perhaps chilly, has slight headache, and sneezes frequently. In severe cases there are pains in the back and limbs. There is usually slight fever, the temperature rising to 101 F. The pulse is quick, the skin is dry, and there are all the features of a feverish attack. At first the mucous membrane of the nose is swollen, "stuffed up," and the patient has to breathe through the mouth. A thin, clear, irritating secretion flows, and makes the edges of the nostrils sore. The mucous membrane of the tear ducts is swollen, so that the eyes weep and the conjunctivae are injected. The sense of smell and, in part, the sense of taste are lost. With the nasal catarrh there is slight soreness of the throat and stiffness of the neck; the pharynx looks red and swollen, and sometimes the act of swallowing is painful. The larynx may also be involved and the voice becomes husky or is even lost. If the inflammation extends to the eustachian tubes the hearing may be impaired. In more severe cases there are bronchial irritation and cough. Occasionally there is an outbreak of labial or nasal herpes. Usually within thirty-six hours the nasal secretion becomes turbid and more profuse, the swelling of the mucosa subsides, the patient gradually becomes able to breathe through the nostrils, and within four or five days the symptoms disappear, with the exception of the increased discharge from the nose and upper pharynx. There are rarely any bad effects from a simple coryza. When the attacks are frequently repeated the disease may become chronic.

#### PRELIMINARY BACTERIOLOGIC STUDIES

Selecting cases that conformed to the type just described—usually within 24 hours of the onset—several platinum loopfuls of nasal secretion were emulsified in sterile salt solution, and varying amounts of this emulsion were used in the preparation of poured human blood-agar plates. One series of plates was incubated aerobically at 37 C., and a duplicate series was similarly incubated under strictly anaerobic conditions. The secretions from 16 cases were examined in this way.

As a rule, the plates prepared from secretions collected within 24 hours of the onset, while the nasal secretion was thin and clear, remained sterile or showed, at most, but few colonies, the identity of which varied in different cases. Many writers note this paucity of bacteria in the nasal secretion from early coryzas. On the other hand, secretions that had become mucopurulent or blood-streaked uniformly

<sup>2</sup> The Principles and Practice of Medicine, Ed. 8, 1916, p. 382.



gave innumerable colonies in plate cultures, but here again there was no uniformity in the findings. In these cases various hemolytic streptococci, *Strep. viridans*, *M. catarrhalis*, and the pneumococcus predominated in numbers in the order named.

I desire to lay emphasis on the negative findings in early cases as challenging the popular belief that the nasal cavities furnish a normal habitat for various bacteria capable of pathogenicity under certain conditions. Numerous workers have shown that the normal nasal mucosa is sterile or practically so. Cecil,<sup>3</sup> in an investigation of *Strep. viridans* in its relation to infections of the upper respiratory tract, found that cultures from coryzas in the acute stage were often sterile, and he states that cultures taken in the subacute or chronic stages often show so many different types that it is difficult to decide which organism is the causative factor. He further found that *Strep. viridans* infections nearly always start in the throat and extend upward or downward, or both.

It became apparent early that nothing tangible was to be gained by resorting further to the usual bacteriologic methods in searching for a specific causative factor, and the problem was approached from another point of view.

#### DEMONSTRATION OF A FILTRABLE VIRUS

Some time ago, Kruse<sup>4</sup> pointed out that while common colds are undoubtedly infectious, the scarcity of bacteria in the secretions and the short time that these few persist militates against the purely presumptive evidence that they are concerned in the infection. In attempting to trace the causative factor by means other than the bacteriologic methods that had been resorted to previously, he succeeded in producing colds experimentally with a filtrate obtained from the nasal secretion of an assistant who was ill with coryza. The secretions blown from the nose were diluted 15 times with normal salt solution, and passed through a small Berkefeld filter. A few drops of the filtrate dropped on the nasal mucosa of each of 12 men produced acute colds in 4, or 33% of the 12. Repetition of this experiment on a more elaborate scale gave confirmation to his earlier results. In the second experiment the nasal secretion was diluted 20 times and, of the 36 students who volunteered for inoculation, 42% became ill with the usual symptoms of coryza within 1-4 days. Kruse states that he was unable to demonstrate living organisms in his filtrates by bacteriologic methods, and concludes that the causative organism should be classified with the filtrable viruses.

During the winter of 1915-1916 I succeeded in confirming Kruse's observations and this fact, together with results that suggested that a living virus had been cultivated in vitro, was communicated in a

<sup>3</sup> The Laryngoscope, 1915, 25, No. 2, p. 97.

<sup>4</sup> München. med. Wchnschr., 1914, 61, 1547.

preliminary note<sup>5</sup> in April, 1916. In the present communication the experimental work briefly outlined in the preliminary report will be reviewed in detail and additional notes, on the cultivation of a minute filterable micro-organism from nasal secretion filtrates, reported.

#### MATERIAL

The first nasal secretion studied was obtained from one of the laboratory staff who had been ill with an acute coryza for two days. He complained of lassitude, chilly sensations, sneezing, unilateral nasal stuffiness, dull frontal headache with a feeling of oppression over the eyes, impairment of smell, and moderate aching pain in the extremities. There was the usual picture of an acute cold: injected conjunctivae, considerable lacrimation, a copious, thin, mucoid nasal discharge which excoriated the upper lip and the alae of the nose, and a very red, moist, swollen and boggy mucosa. The temperature was normal.

The second nasal secretion was collected on the first day of an acute cold experienced by myself. This attack was manifested by symptoms almost identical with those of the first case.

The third specimen was obtained from an employee of one of the large department stores who had been ill for three days. She complained of malaise, feverishness, aching pain in the back, nasal stuffiness, sneezing, frontal headache, dull pain over the eyes, impairment of taste and smell, slight soreness of the throat, and moderate cough with expectoration. The nasal mucosa was moist, red, swollen, and boggy. The nasal secretion was scant and mucoid, and showed admixture of shreds of purulent material. The nasal alae were markedly excoriated and the eyelids were red and puffy. This patient felt certain that she had had considerable fever the preceding evening, but the temperature was normal at the time the nasal secretion was collected.

#### THE PREPARATION OF FILTRATES

In each case the nasal secretion was blown from the nose into a sterile Petri dish, mixed with 10 c.c. of sterile 0.8% salt solution, and carried to the laboratory in a sterile test tube. The material was then poured into a sterile shaking bottle containing smooth round glass beads, and agitated for 10 minutes in a shaking machine. A thoroughly homogenous mixture, free from clumps or strings of mucus, resulted. This mixture was then passed through a small Berkefeld N filter. The filters used in this work were new, their reaction had been neutralized by boiling and repeated washing, and they were impermeable to ordinary bacteria. Filtration was accelerated by suction from a Chapman water pump attached to the laboratory tap. The resulting rate of filtration was such that about 5 c.c. of brilliantly clear filtrate was collected in 15 minutes. Human blood-agar plates prepared from the filtrates and incubated at 37 C., under both aerobic and anaerobic conditions, remained sterile at the end of 7 days.

#### INOCULATION TESTS WITH NASAL SECRETION FILTRATES

Ten soldiers at Fort Banks, Mass., who volunteered for the experiment, were inoculated with the filtrates described. The men were in good health at the time. In each case the nose was mechanically

<sup>5</sup> Jour. Am. Med. Assn., 1916, 66, p. 1180.

cleansed with water, but no predisposing factors to infection, such as irritating or abrading the mucosa, were added. Inoculation was performed by simply tilting the head back so that the material would gravitate into the nose, and the filtrate was dropped into the nasal opening from a capillary pipet. From 3-6 drops of filtrate were placed in each nostril.

CASE 1.—The patient stated that he never remembered having had a cold. All members of his family—wife, sister, and 3 children—had repeated respiratory infections during the winter and, despite intimate daily contact, he remained well. This would indicate a probable high degree of immunity.

He was inoculated with 3 drops of nasal secretion Filtrate B in each nostril, at 9 a. m., February 22.

He was under observation for 4 days. No symptoms occurred.

CASE 2.—There was no history of coryza during the winter. The patient stated that he was not prone to colds. He recovered from an attack of acute tonsillitis a week prior to inoculation.

He was inoculated at 9 a. m., February 22, with 4 drops of nasal secretion Filtrate B in each nostril.

About 8 hours after inoculation his nose became dry and stuffy and by evening he was forced to breathe through his mouth. During the night he sneezed incessantly and "ringing in the ears" developed. The following morning his "head was stopped up," and he complained of slight cough. There was dull frontal headache, with pain over both orbits. The temperature and pulse were normal. When next seen, 48 hours after inoculation, the nasal mucosa was very red, swollen and boggy, and the left nostril was occluded, causing mouth breathing. The voice had a distinct nasal twang. The throat felt very dry, and there was complaint of pain over the frontal sinuses. The temperature was 99 F., and the pulse was 96. Treatment was started.

CASE 3.—There was no history of respiratory infections during the winter, no proneness to colds, and no history of recent exposure.

He was inoculated at 9 a. m., February 22, with 5 drops of nasal secretion filtrate in each nostril. Virus B was used.

The soldier stated that slight nasal discharge started 12 hours after inoculation. The following morning, 24 hours after inoculation, he complained of nasal stuffiness, and had several attacks of sneezing. About 4 p. m. in the afternoon he developed frontal headache, with dull pain over the left eye. The patient stated that he did not feel feverish. The following morning, 48 hours after inoculation, he complained of a slight unproductive cough. The nasal mucosa was dry, glazed, and tense, and there was no secretion. The headache had subsided, and the temperature was normal. Profuse rhinorrhea developed 4 hours later; the cough became worse, and headache returned. On the 4th day the nasal mucosa was still very red, swollen, and boggy, and it was bathed with mucoid secretion. The air space on the right side was occluded by the swollen mucosa. The nasal alae were excoriated. The temperature was 98 F. Except for discomfort occasioned by nasal stuffiness he felt well and on the 5th day all symptoms had ameliorated.

CASE 4.—The soldier was prone to colds. He was inoculated at 9 a. m., February 22, with 6 drops of nasal secretion Filtrate B in each nostril.

The onset of symptoms occurred about 30 hours after inoculation, with a most disagreeable sensation of fulness in back of the eyes. He stated that his nose was "as dry as a bone," and that his throat felt parched. During the night he got up repeatedly to quench his thirst, and flush out his nose. The nasal mucosa was so swollen that he was forced to breathe through his mouth, and he attributed the dryness of the throat to this. When seen again, 48 hours after inoculation, he felt listless and out of sorts, and complained of dull frontal headache, vertigo, and dryness of the nose and throat. The temperature was 99.4 F., and the pulse was 90. On the 3rd day the nasal mucosa was still dry and tense, but lassitude, sensation of fulness in the head, and nasal stuffiness were less troublesome. The lower lip was considerably swollen and covered with herpes. He stated that on arising in the morning he experienced a sharp stitch of pain in the lumbar region, while putting on his shoes. This pain disappeared after he had "limbered up" by walking around for a time. There was complaint of occasional sensations of flushing—"flashes of heat," as he expressed it. The temperature at this time was 99 F., and the pulse was 88. On the 4th day he felt entirely well.

CASE 5.—The patient had 2 attacks of coryza during the winter, the last a month prior to inoculation. He was not unduly prone to colds.

He was inoculated at 9 a. m., February 22, with 5 drops of nasal secretion Filtrate A in each nostril.

The soldier was sent on an ambulance call 24 hours after inoculation and, although the weather was moderate and he was warmly dressed, he had repeated sensations of chilliness during the trip. He developed profuse rhinorrhea 2 hours after returning to the hospital. During the night he complained of dull frontal headache, attacks of chilliness alternated with sensations of flushing, and the nasal alae and upper lip became excoriated from the copious, thin, watery nasal discharge. When seen again, 48 hours after inoculation, he complained of lassitude, "ringing in the ears," slight backache, a sensation of fulness over the frontal sinuses, and moderately dry, unproductive cough. The temperature was 99 F., and pulse was 80. On the 3rd day all symptoms had ameliorated, and the temperature was normal. He still felt languid and the frontal headache had not disappeared entirely. The nasal discharge was less copious and thicker. On the 4th day he complained of slight soreness of the throat and the frontal headache persisted. The temperature registered 99.4 F. The following day, 5 days after inoculation, his only complaint was of stiffness of the muscles of the neck and rheumatoid pains in the right arm. The 6th day he was quite himself again, except for occasional rheumatoid twinges in the muscles of the arm.

CASE 6.—The patient had several colds during the winter. The last attack was 3 weeks prior to inoculation. There was no history of recent exposure.

He was inoculated at 9 a. m., February 22, with 5 drops of nasal secretion Filtrate A in each nostril.

The soldier stated that slight frontal headache and nasal stuffiness developed about 6 hours after inoculation. He slept well and had no symptoms on awakening. About 2 p. m., February 23, he had several paroxysms of sneezing, and the throat felt dry and sore. During the evening moderate mucoid nasal discharge was noted, and headache returned. On the 3rd day the conjunctivae were injected, there was moderate lacrimation, the eyelids were red and puffy, the nasal mucosa was very red, swollen, and edematous, and the upper lip and alae of the nose showed the characteristic irritation from the nasal discharge. The tonsils were slightly swollen and edematous, and showed a few patches of filmy surface exudate. The entire pharynx was reddened. There was no



glandular enlargement, but the left tonsil was palpable externally and tender. Both parotids were tender, but not appreciably swollen, and there was pain on moving the jaw. The face was flushed, and the skin felt dry and hot. The temperature was 99.4 F., and the pulse was 84. On the 4th day the nasal discharge was still copious, frontal headache persisted, and he complained of aching pain in the back and extremities. The temperature was 99.4 F., and the pulse was 90. All symptoms ameliorated on the 5th day.

CASE 7.—The patient was not prone to respiratory infections, and had no cold during the winter. There was no history of recent exposure.

He was inoculated at 9 a. m., February 22, with 6 drops of nasal secretion Filtrate A in each nostril.

The onset was ushered in 26 hours after inoculation with dull frontal headache and attacks of sneezing. This was followed by a sensation of nasal stuffiness and dryness of the throat. In about 3 hours there was copious rhinorrhea and considerable lacrimation. During the evening he had slight tinnitus, and complained of deafness in both ears. He felt feverish, but did not record his temperature. He took 4 "rhinitis capsules" containing belladonna, and nasal secretion was perceptibly checked. He stated that he had several paroxysms of coughing during the night. The cough was dry, harassing, and unproductive. When seen the following morning, 48 hours after inoculation, he was coughing violently; there was a copious mucoid nasal discharge, and he was perspiring freely. The temperature was 99.4 F., and pulse was 88. These symptoms continued during the day and at 6.30 p. m., the temperature registered 100 F. On the 3rd day all symptoms ameliorated and the temperature was normal. Slight cough still persisted. On the 4th day he complained of slight sore throat, but felt well otherwise, and on the 5th day he was convalescent.

An interesting epidemiologic factor in this case is that during the 3rd day of his illness a niece living at his quarters became ill with an acute cold. It was apparently a contact infection.

CASE 8.—The patient gave a history of 2 mild attacks of coryza during the winter. There was no history of recent exposure.

He was inoculated at 9 a. m., February 22, with 6 drops of nasal secretion Filtrate A in each nostril.

Sneezing began about 6 hours after inoculation, and continued at intervals during the evening. There was the usual complaint of dull frontal headache. He slept well and on awakening the following morning his throat felt dry and parched, and nasal breathing was obviated by a sensation of stuffiness. A copious watery nasal discharge containing some mucus was noted during the forenoon, and he had to blow his nose repeatedly in order to breathe through it. Toward evening there were sensations of chilliness—"creeping chills," as he expressed it—but no distinct rigor. Sleep the 2nd night was disturbed by difficulty in breathing through nose. On the 3rd day he looked ill. The conjunctivae were injected, the eyelids were red and swollen, there was much lacrimation, and a copious rhinorrhea caused him to sniff and to use his handkerchief repeatedly, as he was interrogated. The upper lip and alae of the nose were excoriated. There was a slight unproductive cough. The nasal mucosa was very red, and showed marked boggy swelling. He felt listless and out of sorts. The temperature was 99.2 F., and the pulse was 120. Treatment was started and by the 5th day all symptoms had disappeared.



CASE 9.—The patient was prone to respiratory infections. He had adenoids. The tonsils were removed 8 months prior to inoculation. He gave a history of several colds during the winter.

He was inoculated at 9 a. m., February 22, with 5 drops of nasal secretion Filtrate C in each nostril.

The nose and throat became dry about 6 hours after inoculation. The following morning there was a slight nasal discharge which became blood-tinged after repeated blowing of the nose. He said he felt feverish during the day, but his temperature was not recorded. On the 3rd morning he complained of lassitude, and the nose and throat felt dry. There was moderate hoarseness. The temperature was normal. On the 4th day he complained of dull frontal headache, and on the 5th day there was slight sore throat and stiffness of the muscles of the neck. No elevation of temperature was noted.

This case is regarded as questionable because of the catarrhal diathesis of the subject and the reasonable doubt that arises as to whether the slight symptoms following inoculation indicate a true reaction or simply a slight flare-up of a chronic condition. The results lacked the clear cut and definite character of the other inoculation experiments.

CASE 10.—This soldier had several operations on the nasal septum prior to enlistment. The resulting scar tissue caused crust formation and difficulty in breathing. He stated that he was not prone to colds, and that he had not been ill during the winter. He was confined to hospital with a lumbar strain incurred in the gymnasium.

He was inoculated at 9 a. m., February 22, with 4 drops of nasal secretion Filtrate C in each nostril.

The soldier stated that he had several attacks of sneezing about 30 hours after inoculation, and that slight nasal discharge followed. On the 3rd day there was a sensation of fulness in the right ear, with slight impairment of hearing. He felt a little listless, and the nose felt dry. The temperature, recorded morning and evening, remained normal. The symptoms were slight and not clearly defined.

The reaction, if any, in this case was classified as doubtful. There was no febrile reaction, and anatomic defects existed that might readily account for the ill-defined symptoms recorded.

Analysis of the results of these experiments showed that of the 10 men inoculated, 7 developed clear cut and definite symptoms of acute coryza; 2 reacted questionably, while 1 remaining case exhibited no symptoms. There was an incubation period of 6 or 8-30 hours. The initial symptoms, as a rule, were dryness of the nose and throat and attacks of sneezing, dull frontal headache and a sensation of pain or fulness over the frontal sinuses. Several of the men complained of alternate sensations of chilliness and flushing. There was copious rhinorrhea, usually on the 2nd day, in a majority of the cases. Six of the subjects exhibited slight rise in temperature—99.2-100 F.—and in these cases the pulse was accelerated. Tinnitus aurium or slight impairment of hearing was recorded in 4 instances; sore throat in 5; cough in 5, and aching pains in the back or extremities

in 4. One of the men complained of parotid tenderness and pain on moving the jaw; in another case, a marked crop of herpes labialis was noted. The duration of the symptoms varied 3-6 days—usually 5.

During the period preceding these experiments, and while the observations were being made, there was no epidemic of colds in the command. The admission-rate for respiratory diseases was normal. The men were chosen at random as they volunteered and their duties, hygienic conditions, and general environment differed in no way from those of the remaining 250 men of the garrison. It would appear, therefore, that the experiments were adequately controlled.

#### CULTIVATION EXPERIMENTS

The results of the inoculation experiments with nasal secretion filtrates, and the sterility of these filtrates as regards micro-organisms capable of demonstration by ordinary bacteriologic methods, seemed to indicate clearly that the infective agent is a filtrable virus. The successful cultivation of the parasite of rabies by Noguchi,<sup>6</sup> and the causative micro-organism of poliomyelitis by Flexner and Noguchi<sup>7</sup>—both organisms being “filter passers”—suggested the application of methods similar to those of Noguchi in attempting to grow an organism from the nasal secretion filtrates.

Several mediums were used in the earlier experiments and of these the most suitable has proved to be tissue-ascites fluid. About 12 c.c. of ascites fluid is poured into each of several test tubes measuring  $1 \times 20$  cm., and a small piece of sterile fresh rabbit kidney is placed in the bottom of each tube with strict aseptic precautions. About 4 c.c. of sterile liquid petrolatum is then poured on the fluid medium and the tubes incubated for several days at 37 C., and at room temperature to insure their sterility. After a day or so, some of the blood from the kidney undergoes hemolysis and imparts to the medium surrounding the fragment of tissue a rose-colored tint; but in tubes that are not contaminated, this zone of hemolysis remains brilliantly clear. Any tubes showing haziness or cloudiness should be discarded.

After 18 months' experience with this technic, involving the examination of several hundred cultures prepared from many samples of ascites fluid and the tissue from many rabbits—not only in the work to be reported but in other studies—I am convinced that one should approach this work with the greatest patience and care, as successful results depend largely on the technical considerations involved. A visit to the Rockefeller Institute cannot help but impress one with the reasons for Noguchi's success in cultivating organisms that had previously proved refractory; and one should hesitate in attacking such work, because of his inability to confirm it, unless he has facilities equal to those of the original workers and considerable preliminary experience with the technic.

<sup>6</sup> Jour. Exper. Med., 1913, 18, p. 314.

<sup>7</sup> Ibid., p. 461.

The choice of a satisfactory ascites fluid is of prime importance. Certain workers have given standards by which the fluids may be judged, such as specific gravity, color, fibrin content, and the presence or absence of bile. In my work I have not depended on any of these considerations, as it is conceivable that a fluid that offers a satisfactory medium for some micro-organisms may not be suitable for the growth of others. Each attempt should be conducted with mediums made from several different ascites fluids, and when by actual test one is found that proves satisfactory, it should be carefully husbanded in the cold-room for future use. The ascites fluid should be originally sterile, and not subsequently sterilized by filtration or other methods.

Healthy rabbits should be chosen to furnish the tissue fragments used. It is my custom to strap the rabbit to an animal-board, cut the hair from the entire thorax and abdomen with scissors, and remove the remaining short hairs with a razor or a strong solution of sodium sulphite. The animal is stunned by a blow on the head, and a sharp bistoury is passed through the tissues of the neck so as to sever the carotid and jugular. The foot of the board is raised, and the body quickly becomes drained of blood. Death is instantaneous and painless. The entire abdomen is painted with a thick coating of tincture of iodine. The body of the animal and the board to which it is strapped are then enveloped in sterile towels saturated with a strong solution of liquor cresolis compound, leaving the portion of the abdomen that has been painted exposed, and taken to a dust-proof room where the remainder of the procedure is carried out. Gauze is tied over the nose and mouth of the operator and his assistant as a precautionary measure against contamination. All instruments used in the operation are sterilized by dry heat at 170 C., for at least 2 hours. They are brought to the operating room in the unopened pipet box in which they were sterilized. A median incision is made through the skin with a scalpel from the upper part of the sternum to the pubic region and, grasping the edges of the incised skin with rat-tooth forceps, it is dissected back and held out of the way with artery forceps. The instruments used thus far are now discarded. Next a spatula is heated to redness, and by repeated applications a semilunar scar is made on the exposed tissues, extending from the left inguinal region to the lower border of the ribs on the same side, its convexity being over the right side of the abdomen. The tissues underlying the lower part of the scar are picked up with forceps, and the abdomen is opened by cutting through the seared line in its entire extent. The resulting semilunar flap is turned back and held out of the way with a clamp. The intestine is now lifted out of the way and a kidney exposed. With fresh forceps the kidney is quickly lifted from its bed and transferred to a large sterile culture dish. The capsule is stripped from the kidney with forceps and the organ is then cut into 12-15 pieces with small sterile scissors. The assistant holds the cover of the dish over the field during this procedure. The remaining kidney is disposed of, of course, in the same manner. The fragments of tissue are then transferred to the culture tubes with long slender forceps or, if these are not at hand, by picking them up on a hot platinum loop to which they adhere. The test tubes are sterilized by dry heat and the necks are flamed just before and just after adding the ascites fluid and the tissue. The liquid petrolatum that is superimposed on the medium is sterilized in the autoclave under 15 pounds pressure, on 2 successive days.

Rigid adherence to this technic results in exceedingly few contaminations. Occasionally, after incubation, a sample of ascites fluid shows contamination with a small gram-positive coccus; but all the tubes will show the same organism, and one cannot be misled if each culture is controlled by tubes of unin-

oculated medium incubated with the cultures. Another occasional source of contamination is a small pleomorphic diphtheroid organism that apparently comes from the tissue. In each experiment the sterility of the medium should be insured by incubation prior to inoculation, and the procedure further safeguarded by incubating control tubes of uninoculated medium with the inoculated tubes.

Sterile capillary pipets, with the capillary end drawn out to a length of about 25 cm., are used in inoculating the tubes. The pipets are plugged with cotton, placed in large glass tubes plugged with cotton, and sterilized at 160 C., for at least 2 hours. The pipets are removed with care, the capillary end is flamed, and a rubber nipple is fitted to the proximal end. About 0.5 c.c. of the nasal secretion filtrate is drawn into the pipet and, being careful that no air bubbles are introduced below the layer of oil, the tip of the pipet is thrust through the medium to the bottom of the tube, and the filtrate introduced into that part of the medium surrounding the tissue. The introduction of air bubbles at the end of the procedure may be prevented by not entirely emptying the pipet of its contents. Originally, the cultures prepared in this way were incubated in an anaerobic jar. Later experience has shown that this is unnecessary as an adequate degree of anaerobiosis is occasioned by the fragment of tissue in the medium. In making initial cultures it is well, however, to incubate one series of cultures in an anaerobic jar and a duplicate series outside the jar.

#### MACROSCOPIC APPEARANCE OF CULTURES

An initial culture has now been obtained in 11 cases of acute coryza acquired in the usual way, and from 5 cases induced by experimental inoculation. The initial cultivation is attended with more or less difficulty; but once the growth has been established the virus tends to become saprophytic, and subsequent cultivation is attended with less difficulty. I have not succeeded in obtaining an initial growth in solid mediums. Subculture in solid mediums is possible, however, after growth for 2 or 3 generations in the fluid medium.

The appearance of initial cultures in tissue-ascites medium varies in degree with the various filtrates used as inocula, but is otherwise always the same. A distinct, filmy, grayish white, opalescent halo, superimposed on a cloudy hemoglobin-stained zone surrounding the tissue, is observed at the end of 24 hours. This halo or ring is sharply demarcated from the clear fluid above, and shows no tendency to blend with it. The appearances are not unlike those resulting from the addition of nitric acid to urine containing a trace of albumin. It is probable that this is partly due to chemical changes of a protein nature in the ascites fluid, induced by the virus or constituents of the secretion filtrate. At the end of 48 hours the changes are at least 100 per cent. more marked, while on the 3rd day the tissue is more or less obscured by the density of the cloudiness surrounding it. On the 4th day the upper column of fluid, which has heretofore remained clear,



shows slight uniform haziness which increases in density as time goes on. In old cultures the upper column of fluid becomes clear again, and a finely granular or powdery sediment collects around the tissue in the bottom of the tube.

Control tubes inoculated with salt solution show a very faint halo at the end of 24 hours, similar to that observed in the culture tubes; but there is no cloudiness in the zone surrounding the tissue, and the change does not become intensified on subsequent days. After 2 weeks' incubation some autolysis of the tissue becomes evident, and there is slight haziness of the supernatant fluid due to admixture of minute particles of autolyzed tissue.

The changes in tissue-ascites subcultures are more marked than in the initial cultures, and the cloudiness in the tissue zone exhibits more tendency to extend upward and diffuse into the clear medium.

In making subcultures in the solid medium, 0.5 c.c. of the Berkefeld filtrate of the subculture to be transferred is added to a sterile tube containing a piece of sterile rabbit kidney. A mixture of equal parts of ascites fluid and 2 per cent. nutrient agar is then added, and 3 or 4 c.c. of liquid petrolatum are superimposed on this. The agar is boiled to drive off the oxygen, and then rapidly cooled to 42 C. The ascites fluid is brought to the same temperature as the agar before admixture with it. The appearances of growth in this medium are similar in a way to those in the fluid medium. The growth appears in 4 or 5 days as a faint grayish film surrounding the tissue. Individual colonies are not discernible at this stage, even with the aid of a lens. The growth gradually extends upward and diffuses into the clear medium for a distance of 4 or 5 cm., the upper limits of the haziness blending imperceptibly with the clear medium. There is no tendency toward demarcation of the growth, such as occurs in the fluid medium. After several days' growth extremely minute colonies that may be brought out with a lens are demonstrable in the zone immediately above the tissue. These colonies occur only in this limited zone, and here the growth is most dense. The growth in the solid medium is much slower than in the tissue-ascites medium, and I have been able to obtain the growth only in subcultures after cultivation for 2 or 3 generations in the fluid medium.

#### DEMONSTRATION OF A MICRO-ORGANISM IN CULTURES

In my preliminary communication<sup>5</sup> I stated that stained preparations from the cultures showed no bodies that could be "definitely recognized" as micro-organisms, although "minute coccoid bodies and occasional rodlike forms were demonstrable in all the preparations, but not in sufficient numbers to preclude the possibility that they were artefacts." The original cultures were examined on the 3rd or 4th day, and only the uppermost limits of the macroscopic growth was examined. Shortly after submitting this report for publication addi-



tional preparations were studied from cultures that had been incubated 14 days, the material pipetted from the bottom of the tubes in the zone surrounding the tissue. Innumerable coccoid bodies were demonstrable in these preparations.

Subsequent experience has shown that the original failure to demonstrate the micro-organism in unmistakable numbers was due to the preparations having been made from early cultures, before appreciable multiplication had occurred, and to the selection of a zone of the cultures where, at best, growth is scant. This oversight was occasioned by a deliberate effort to avoid the zone surrounding the tissue, as it was thought that products of tissue autolysis might give rise to confusing artefacts.

Stained preparations from cultures that have been incubated 7-14 days—the period during which maximum growth occurs—show extremely minute coccoid bodies occurring singly, in pairs, and in agglomerations of varying size. Occasionally there appears to be slight tendency to chain-formation. The chains are not numerous and they are short, consisting of 4-6 elements. The bodies show marked variation in size, but are uniformly coccoid or globular in shape. Forms just approaching visibility, bodies larger than a staphylococcus and intermediate forms exhibiting all gradations in size are demonstrable frequently in a single group. The larger forms often show minute bodies adherent to them, which suggests that multiplication occurs by budding. The predominating type is uniformly the small globoid form measuring 0.2-0.3 of a micron in diameter.

The micro-organism stains well with Giemsa's stain, and less clearly with the ordinary laboratory stains. The bodies in young cultures are gram-negative. In old cultures some of the bodies, especially the larger forms, are variable in their behavior toward the gram stain, retaining the gentian violet, or only becoming decolorized after repeated washing with alcohol.

The most satisfactory results have been obtained with Giemsa's stain. The preparations are air-dried without heating, fixed in absolute methyl alcohol for 1 hour, washed in water, and then stained over night in a Coplin jar containing Giemsa's solution in the proportion of 1 large drop of the stain to each cubic centimeter of distilled water. To avoid artefacts any excess of stain is removed by immersing the preparation in acetone for a few seconds, and then washing thoroughly in distilled water. A majority of the bodies are stained a distinct purple by this method. In old cultures, however, there is more or less variability in the color, some staining deep blue and others reddish. Varying degrees of intensity of staining are also evident in old cultures, the extremely minute, and very large forms staining faintly, while the intermediate forms are colored a deep blue. Some of the bodies stain poorly and appear to be inclosed in a more deeply staining peripheral structure. It is not certain whether this indicates the possession of a true capsule or a 'pseudo-capsule' composed of albuminous material from the culture medium. Most of the tinctorial and morphologic characteristics are evident in the photomicrographs (Figs. 2, 3, and 4.)

It becomes evident at once that this micro-organism differs markedly from any known organism with the possible exception of the

"globoid bodies" described by Flexner and Noguchi,<sup>7</sup> and believed by them to be the causative factor in poliomyelitis. An extremely pleomorphic streptococcus, the minute forms of which bear some resemblance to this micro-organism, has recently been described in connection with poliomyelitis, also, by Mathers,<sup>8</sup> Rosenow, Towne and Wheeler,<sup>9</sup> and Nuzum and Herzog.<sup>10</sup>

Through the courtesy of Dr. Noguchi and Dr. Rosenow, I have been able to study preparations of the micro-organisms described by them and to compare these with my own preparations. The slide received from Dr. Rosenow shows an extremely pleomorphic coccus ranging in size from elements that just approach visibility to forms fully as large as a pneumococcus. The arrangement is in clumps, as a rule, with a very definite tendency to chain-formation. The pleomorphism observed in this preparation is striking. The predominating form is coccoid in shape, stains with moderate intensity, and is arranged in groups and chains. A moderate number of large cocco-bacilloid forms are observed, occurring singly, in pairs, and in short chains of up to 8 elements. These forms have a lightly stained zone across the lesser diameter and the ends stain deeply, giving them the appearance of coccobacilli. Numerous bacilloid types also occur. Of these some exhibit polar staining, others appear fusiform, and bizarre types with clubbed ends and sporelike bodies also occur. Dr. Noguchi's preparation shows small globoid bodies that are uniform in size and shape. These bodies are extremely minute, measuring 0.2-0.3 of a micron in diameter. They occur singly, in pairs, in short chains, and in groups. Occasionally the more deeply stained elements appear to be larger than the remainder, but there is no marked variation in size. In common with these 2 poliomyelitis types, the micro-organism isolated in common colds shows as its predominating type minute coccoid or globoid bodies arranged in groups. Unlike the Flexner-Noguchi bodies, however, there is considerable variation in the size of the bodies, and some of the larger ones show possible budding. While the coccoid bodies resemble the smaller forms observed in Dr. Rosenow's preparation, there is no approach to the pleomorphism seen in the latter—variations in the cold organism being limited to differences in size—and cocco-bacilloid forms do not occur. Furthermore, there is not the tendency to streptococcus types that is so characteristic of the Rosenow organism.

The cultural requirements of the micro-organism isolated in common colds are similar to those of the globoid bodies of poliomyelitis, initial cultivation being obtained with difficulty, and only in the selective Noguchi medium; growth is slow—the micro-organism not being demonstrable in numbers before the 6th or 7th day—and never luxuriant; and cultivation in solid mediums is not possible until the organism has become more or less saprophytic by growth for several generations in the fluid medium. Again, cultivation has not been possible under aerobic conditions. On the other hand, the streptococcus

<sup>8</sup> Jour. Am. Med. Assn., 1916, 67, p. 1019.

<sup>9</sup> Jour. Am. Med. Assn., 1916, 67, p. 1202.

<sup>10</sup> Jour. Am. Med. Assn., 1916, 67, p. 1205.

described by Rosenow and his co-workers is cultivated with comparative facility, and growth is luxuriant on a variety of mediums under both aerobic and anaerobic conditions, but always best under aerobic conditions.

#### FILTRABILITY AND INFECTIVITY OF CULTURES

The inoculation experiments with nasal-secretion filtrates, previously cited, indicate that the virus of common colds is filtrable, and the interesting questions now arise as to whether the virus is capable of being cultivated *in vitro*; whether the appearances of macroscopic growth observed in cultures are in reality due to the multiplication of this virus; and, finally, whether the minute micro-organism that has been described bears any relation to the true infective agent.

Experimental evidence has been obtained that should leave little doubt that the virus is capable of cultivation, and that the virus so cultivated is infective after passage through a Berkefeld N filter. Additional experiments strongly suggest, although conclusive proof has not been obtained, that the minute micro-organism demonstrated in cultures is the true infective agent. This micro-organism has been demonstrated in subsequent generations of subcultures that had proved infective by inoculation experiments; and it has been proved that the micro-organism is filtrable by the passage of fluid medium cultures through Berkefeld N filters and the subsequent demonstration of the micro-organism in both fluid and solid mediums inoculated with the culture filtrates.

The inoculation experiments that follow, however, were made early in the investigation—during March, 1916—and, due to the technical oversight that has been mentioned already—examining the cultures too early and selecting a zone where growth is scant—micro-organisms in unmistakable numbers were not demonstrable. In subsequent subculture generations of the 2 viruses used, however, the minute micro-organism was demonstrated unmistakably.

Subcultures of the nasal secretion filtrate Viruses A and B, that had proved infective before cultivation in the first series of inoculation experiments, were used in the experiments that follow. These subcultures were of the 2nd generation. Portions were diluted 10 times with sterile normal salt solution and passed through Berkefeld N filters. The resulting filtrates were perfectly clear and proved sterile as regards organisms capable of growth on aerobic and anaerobic blood-agar plates.

By dilution of the nasal secretion with salt solution, the further dilution occasioned by the fluid-culture mediums, and the final dilution of the portions of subcultures used with sterile salt solution, the constituents of the original nasal secretions that were capable of filtration had reached, in these filtrates, a dilution of 1:90,000 plus the unknown original dilution occasioned by the fluids of the nasal secretion itself.

Eleven soldiers voluntarily submitted to inoculation with these subculture filtrates. The technic of inoculation was identical with that used previously in the experiments with nasal secretion filtrates. Five drops of the diluted culture-filtrate were dropped into each nostril from a sterile capillary pipet.

CASE 1.—The patient was not prone to colds, although he usually had 1 or 2 attacks during the winter. He had not had a severe cold during the present season.

He was inoculated at 8:30 a. m., March 3, with 5 drops of diluted subculture-filtrate of Virus A in each nostril. The filtrate was 18 hours old.

At 2:30 p. m., 6 hours after inoculation, he experienced chilly sensations, followed by unilateral nasal stuffiness and a sensation of fulness over the frontal sinuses. The temperature at 5 p. m., was 99 F., and the pulse was 80. The following day the nasal mucosa was swollen and boggy. There was slight nasal discharge. The only subjective symptoms were nasal stuffiness and attacks of sneezing. The temperature was 98.2 F., and the pulse was 72. On the 3rd day all symptoms had ameliorated.

CASE 2.—The patient very seldom had a cold. He had not been ill during the winter.

He was inoculated at 8:30 a. m., March 3, with 5 drops of diluted subculture-filtrate of Virus A in each nostril.

The soldier stated that he developed severe frontal headache and dryness of the nose and throat about 8 hours after inoculation. There was a sensation that the nose was running, but no secretion was evident. At 5 p. m., he complained of difficulty in breathing through the nose, and examination showed the mucosa to be much swollen. The temperature was 98.8 F. The patient was not seen again, and there is no record of subsequent symptoms.

CASE 3.—The patient stated that he was moderately susceptible to infection. There was history of an acute cold 2 months prior to inoculation.

He was inoculated at 8:30 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus A in each nostril.

When seen at 5 p. m., 8 hours after inoculation, he stated that he had had several paroxysms of sneezing, and he complained of slight unproductive cough. Moderate rhinorrhea was evident, and the nasal mucosa was much swollen and very red. The temperature was 99 F., and the pulse was 76. The following day the nasal secretion was more copious. On the 4th day all symptoms had disappeared.

CASE 4.—The patient was not susceptible to colds.

He was inoculated at 8:30 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus A in each nostril.



At 5 p. m., on the day of inoculation he had slight frontal headache. There was also complaint of dryness of the throat, and a burning sensation in the nose when breathing. The mucosa was swollen, and there was slight nasal discharge. Twelve hours after inoculation he felt feverish, but the temperature was not recorded. The day following inoculation there was complaint of nasal stuffiness, he was hoarse, and there was a slight cough. The nasal mucosa was much congested, swollen, and moderate rhinorrhea was evident. The temperature was 98 F. Later in the day he had several attacks of sneezing. On the 3rd day there was copious rhinorrhea. Symptoms began to ameliorate on the 4th day, and had entirely disappeared by the 6th day.

CASE 5.—The patient recovered from an acute cold a week prior to inoculation. He was selected for inoculation to test immunity.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus A in each nostril.

The soldier stated that during the evening of the day of inoculation there was slight headache. On the 2nd day the headache still persisted, and there were several attacks of sneezing. During the evening of the 2nd day there was copious rhinorrhea, the conjunctivae were injected, and there was considerable lacrimation. The temperature was normal. Symptoms continued another day, and then rapidly ameliorated.

CASE 6.—The patient stated that he is not susceptible to infection. He had not had a cold for several months.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril. He returned to Fort Andrews after the inoculation and notes on the course of subsequent events were furnished by Sergeant 1st Class Crampton, Hospital Corps.

Twenty-four hours after inoculation the mucous membrane of the nose was red and swollen, and the patient was breathing through his mouth. He complained of nasal stuffiness and slight frontal headache. The headache ceased about 2:30 p. m., and its cessation was followed by the appearance of a thin, clear, nasal discharge. The temperature was 99.2 F. The following day the nasal secretion had diminished, and had ceased by evening.

CASE 7.—The patient stated that he had a cold a month prior to inoculation, but that he was not susceptible as a rule.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril. He was returned to his station and subsequent events were noted by the post surgeon.

A nasal discharge was noticed about 8 hours after inoculation with attacks of sneezing and coughing. The temperature remained normal. The nose felt "stopped up" and he complained of a burning sensation over the eyes. The following morning he still complained of nasal stuffiness, and there was profuse, thin, nasal discharge and considerable cough. The temperature was 99 F. On the 3rd day there was slight difficulty in breathing through the nose, and slight cough persisted. On the 4th day he was well.

CASE 8.—The patient stated that he had not had a cold for years.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril. He returned to his station after inoculation and recorded his own symptoms.

On the evening of the day of inoculation he felt "as if he were taking a cold." There was a slight mucoid nasal discharge, and the throat felt dry. No rise in temperature was recorded. An abortive attack occurred.



CASE 9.—The patient recovered from an experimental cold induced with nasal secretion filtrate a few days prior to inoculation. He was inoculated again to test immunity.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril.

During the afternoon he had several attacks of sneezing, and toward evening dull frontal headache, with a sensation of nasal stuffiness, developed. The temperature was normal. The following day there was copious rhinorrhea and cough. The temperature was still normal. The 3rd day all symptoms had disappeared.

CASE 10.—This soldier had been inoculated, also, with nasal-secretion filtrate prior to this experiment.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril.

Eight hours after inoculation he complained of dull pain over the eyes, and the neck felt stiff. The temperature was 99.4 F., and the pulse was 84. The following day he complained of dizziness, dull frontal headache, and aching pains in the back and extremities. The nose and throat felt dry and parched. The temperature was 97.6 F., and the pulse was 72. The symptoms gradually ameliorated and by the 5th day he felt well.

CASE 11.—The patient had recently recovered from a severe cold induced by experimental inoculation with nasal-secretion filtrate.

He was inoculated at 10 a. m., March 3, with 5 drops of diluted subculture filtrate of Virus B in each nostril.

During the afternoon of the day of inoculation he had 2 attacks of sneezing; there was slight rhinorrhea and dull frontal headache. At 5 p. m., the temperature was 99.4 F., and the pulse was 80. The following day he felt out of sorts, complained of dull aching pains in the back and extremities, his throat was dry and sore, there was a slight mucoid nasal discharge, and the afternoon temperature was 99.2 F., and the pulse was 96. The 3rd day the symptoms were the same as on the 2nd. On the 4th day the nasal discharge was profuse, and the alae of the nose and upper lip had become excoriated. He sneezed frequently, the eyelids were red and puffy, and he "looked ill." The temperature at this time was 99 F. The symptoms gradually ameliorated from this time, and by the 7th day he had entirely recovered.

These experiments show that, after incubation periods of approximately the same duration as those occurring in the earlier experiments with nasal-secretion filtrates, unmistakable symptoms of acute coryza were exhibited by the subjects. In one case the attack was mild or abortive, but in the remaining 10 cases the symptoms were well-defined and persisted for several days. The results in Cases 5, 9, 10, and 11, although in accord with clinical and epidemiologic knowledge of the disease, are interesting in their bearing on the questions of immunity and specific therapy. The patient in Case 5 had recently recovered from an acute cold acquired naturally, and the patients in Cases 9, 10, and 11 had recovered but a few days previously from colds induced by experimental inoculation; yet, all of these subjects proved sus-

ceptible to inoculation with the subculture-filtrates. These results suggest that immunity of even short duration is not conferred in this condition, and that specific vaccine therapy would be of questionable value, even were the virus readily cultivable.

Berkefeld filtrates were prepared from the nasal secretions in 5 of these experimental cases, 48 hours after inoculation. Tubes of tissue-ascites medium were inoculated with 0.5 c.c. each of the filtrates, in the manner previously described. Four of the cultures showed the characteristic changes observed in previous cultural experiments. Cultures in the 5th case remained unchanged at the end of 24 hours, but another nasal secretion filtrate from this case, collected 72 hours after inoculation, gave positive results.

The minute micro-organism that has been described was not demonstrated in the initial cultures from these cases. It was demonstrated subsequently, however, in the 3rd subculture generation of the virus from one of the patients (Case 11). The micro-organism has been demonstrated, also, in subcultures of Viruses A and B that were used in both series of inoculation experiments, and in the initial cultures from the nasal secretion filtrates of 7 cases. It has never been encountered in control tubes of uninoculated medium which were uniformly incubated with each series of cultures.

#### DISCUSSION

The experimental evidence that has been presented appears to show that common colds are infectious; that the causative virus occurs in the nasal secretions, and that this virus is capable of passing through Berkefeld N filters that are impermeable to ordinary bacteria. While many of the known organisms that have been described in connection with the causation of common colds, such as the pneumococcus and various streptococci, may act as secondary invaders, prolonging or intensifying the attack or acting as the true cause of numerous complications or sequelae, it is believed that the evidence supports the probability that the primary infective agent is the filtrable virus that has been described.

The question arises as to whether the symptoms following inoculation with this virus might not be explained by the action of a preformed toxin or enzyme in the nasal secretions. Such an explanation is believed to be untenable in view of the distinct latent period that uniformly elapsed between inoculation and the onset of symptoms, together

with the occurrence of slight fever in a majority of the cases. As far as is known, inoculation with toxin is followed by almost immediate reaction, and the symptoms are transitory and never prolonged. The existence of this silent or latent incubatory period also serves to dismiss anaphylactic phenomena from consideration.

The cultural experiments suggest that a living virus has been cultivated *in vitro*, and that the virus so cultivated is infective in at least the first subculture generation. The filtrable constituents of the original nasal secretions were in a dilution of 1:90,000 + in the filtrates of subcultures which produced colds experimentally; and it is inconceivable that any toxin, virus, or enzyme, preformed in the secretions, could have been carried over mechanically and, in this high dilution, exhibited pathogenicity. Such pathogenicity is common only to living bodies capable of growth and multiplication. Secondly, the cultures showed changes that ordinarily are interpreted as indicative of micro-organismal growth, while identical changes were not observed in control tubes. Again, the changes observed in subcultures were intensified in each successive subculture generation, thus bearing an inverse relation to the dilution of the inoculum originally introduced. This phenomenon frequently occurs during the continuous cultivation of known organisms, as the result of acquired adaptability to the conditions in an artificial medium. Finally, minute coccoid bodies have been demonstrated in initial cultures from the filtered nasal secretions of persons ill with acute colds; in subsequent generations of subcultures that had proved infective by inoculation experiments; and in subsequent generations of a culture from the filtered nasal secretion of a person with the symptoms of an acute cold induced experimentally. These bodies have never been encountered as ordinary saprophytes, nor have they been demonstrated in control tubes of uninoculated medium incubated with each series of cultures. The bodies are capable of passing through Berkefeld N filters, as is shown by demonstrating the bodies in cultures and then recultivating identical bodies from the culture filtrates. Because of the extreme minuteness and, at times, indeterminate morphology of many of the bodies, doubt may arise as to their true micro-organismal nature. Any such doubt should be dispelled by the fact that the bodies finally have been grown in a solid medium which obviates the entrance of artefacts that might occur in fluid mediums.

In the absence of conclusive experimental proof, interesting theories suggest themselves as to the relation of the minute micro-organism

described to the true infective agent. It is conceivable that this visible micro-organism might co-exist in cultures with an ultramicroscopic virus, the latter being the true infective agent; but, as pointed out by Flexner and Noguchi<sup>7</sup> in discussing this same question as applied to the micro-organism causing poliomyelitis, an instance of symbiosis of this nature is not known to animal pathology. On the other hand, recent studies in cerebrospinal meningitis, conducted by the English investigators, Hort, Lakin, and Benians,<sup>11</sup> raise the question whether certain micro-organisms do not exhibit a complex life cycle somewhat similar to that which obtains among the protozoa and which appears to obtain among the spirochetes. These workers believe that the meningococcus as it occurs in the cerebrospinal fluid and in cultures is not the true causative factor in epidemic cerebrospinal meningitis. The results of their cleverly conceived and carefully executed experiments suggest that the true infective agent is a filtrable virus and that the meningococcus, as we know it, although not a genuine secondary invader, probably represents a late phase in the life history of an unidentified minute micro-organism that is the true infective agent. In support of this theory they showed that it was possible, under favorable conditions, to demonstrate in the cerebrospinal fluid of acute human cases of epidemic meningitis the presence of a pathogenic virus capable of passage through Chamberland F bougies. The filtrates when injected intraperitoneally into monkeys caused continued fever and death. There was a definite incubation period which should preclude the possibility that the febrile reaction was induced by preformed pyrogenic toxins. As opposed to this, they showed that the injection of other monkeys with cerebrospinal fluid taken from acute cases of the disease produced no effect, notwithstanding the fact that living meningococci were cultivated in numbers from the fluid injected.

In a subsequent paper Hort and Caulfeild<sup>12</sup> report the results of further experiments and conclude that the pathogenicity to monkeys of cerebrospinal fluid from acute cases of cerebrospinal fever appears to tend to vary inversely as the meningococcal content; that the cerebrospinal fluid in this disease contains a filter-passing agent that is not the meningococcus, but which is capable of producing in monkeys continued fever and death; that the filtrable agent is a living virus capable of cultivation and of passage through monkeys, and that the patho-

<sup>11</sup> Jour. Roy. Army Med. Corps, 1916, 26, No. 2.

<sup>12</sup> Ibid., 27, p. 312.



genicity of the meningococcus, as such, appears to be due to the concomitant presence of the filter-passing virus described.

If one reverts to the description that have been given of the morphology and cultural characteristics of the micro-organism isolated in common colds, it is evident that certain facts lend themselves to serious consideration in connection with the theory advanced by Hort and his co-workers. In the first place, the cultures show changes indicative of macroscopic growth several days before visible micro-organisms are demonstrable in numbers, the latter only appearing after cultures have been incubated for 7-14 days. Secondly, the micro-organisms that are demonstrable at first are extremely minute and scant in numbers. Later, as multiplication occurs, larger forms appear, many of which seem to be budding, while others appear to be inclosed in a peripheral structure. Finally, in the older cultures, the micro-organisms show all gradations of size from bodies that are just visible under a high power lens to bodies larger than a staphylococcus.

Is it not conceivable that the gradations of size do not stop with the bodies that are just visible, but that myriads of still more minue forms may occur, causing the early cloudiness in cultures that is out of proportion to the number of demonstrable micro-organisms; and that these possible ultramicroscopic forms represent an early phase of a complex life cycle during which the micro-organism is infective, while the larger bodies, which are visible under the microscope, represent a resting stage in the life history?

Another question that arises is whether the coccoid bodies may not represent mutations of one of the more commonly known organisms. Dr. E. C. Rosenow has informed me that pneumococci placed under cultural conditions similar to those governing the growth of the micro-organism isolated in common colds have shown pictures similar to those seen in preparations of the minute micro-organism described, particularly after filtration of cultures which have shown every gradation from just visible bodies to large diplococcus forms. In view of the uniformity with which pneumococci occur in the mouth and throat, and their frequent presence in the secretions in the later stages of common colds, it is possible that there may be some relation between the bodies described and the pneumococcus. On the other hand, these bodies never become aerobic, even after repeated cultivation; the employment of a special method is required for their cultivation, and



growth does not occur in mediums that furnish pabulum for the pneumococcus. These facts militate against the assumption that there may be a relationship between the micro-organisms.

No opinion is hazarded as to the classification of the peculiar micro-organism that has been described. Moreover, a definite statement at this time in regard to its real relation to the causative agent of common colds would, in the absence of further proof, be based on questionable premises. The fact remains that a very interesting micro-organism has been isolated from the filtered nasal secretions of persons ill with common colds, and sufficient evidence that it bears some relation to the true infective agent has been adduced to merit further investigation.

#### CONCLUSIONS

From the experimental evidence presented it seems that the following facts have been established:

Common colds of the ordinary type are infectious.

The ordinary bacteriologic methods that have been resorted to, heretofore, do not furnish reliable criteria on which to base conclusions as to the etiology of these affections. Cultures made from the nasal secretions early in the acute phase often remain sterile, while cultures made later in the attack frequently show such a diversity of organisms that only presumptive evidence exists for ascribing to any one an etiologic rôle.

It has been demonstrated experimentally that the virus of common colds occurs in the nasal secretions; and that this virus is capable of passing through Berkefeld filters which are impermeable to ordinary bacteria.

By the employment of special anaerobic methods the virus of common colds has been cultivated *in vitro*, and has proved capable of repeated recultivation in subcultures.

Experimental inoculations have demonstrated that Berkefeld N filtrates of subcultures of the virus, in the 2nd generation at least, are infective.

A peculiar minute micro-organism has been isolated from cultures made from the filtered nasal secretions in common colds. This micro-organism can be passed through Berkefeld N filters, and has been recultivated from culture-filtrates. Although conclusive proof of its nature has not been adduced, the experiments suggest that the micro-organism described bears a definite relation to the true infective agent.

## PLATE 9

FIG. 1. Macroscopic appearances of tissue-ascites medium cultures of the virus of common colds. Cultures incubated 7 days. The tube to the extreme left is a control tube that was incubated with the cultures.

FIG. 2. Appearances of the minute coccoid bodies isolated from a 14-day culture. Note the variation in size and the intensity with which the stain is taken. Giemsa stain.  $\times 1000$ .

FIG. 3. Large groups of the coccoid bodies in a 14-day culture. Giemsa stain.  $\times 100$ .

FIG. 4. Selected fields seen in culture preparations of the micro-organism isolated in common colds. Note the occurrence of single bodies and the arrangement in pairs, short chains, and small groups. The upper right field illustrates the appearance of the large budding forms that have been described as occurring in old cultures. The lower right field shows a group of bodies that appear to be inclosed in a peripheral structure. Giemsa stain.  $\times 1000$ .

PLATE 9



Fig. 1

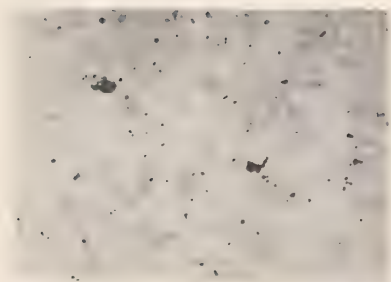


Fig. 2.

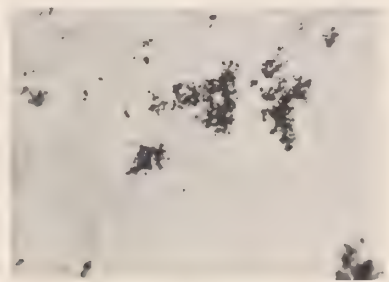


Fig. 3

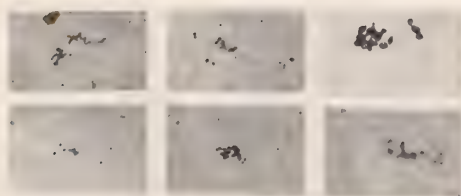


Fig. 4.



# SOME OBSERVATIONS ON HEMOLYSIN- AND ACID- PRODUCTION BY STREPTOCOCCI \*

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Although the hemolysin- and acid-production of streptococci from various sources have been studied by several investigators, we cannot overlook the fact that many important points pertaining to these characteristics remain to be decided more accurately. For instance, to what extent is the acid-production by streptococci in various carbohydrates to be regarded as a definite stable characteristic of certain strains, and what difference is there between a hemolytic halo and a greenish colorization of streptococcal colonies from the biochemical point of view?

The opinions of different investigators on these questions are not exactly coincident. Furthermore, there are various doubtful points with regard to the nature of the growth of streptococci, which should be determined more precisely. In the work included in this communication an attempt has been made to determine whether there is a contradictory relation between hemolysin- and acid-formation in glucose broth. To put the problem more clearly: On the one hand, it is a well known fact that hemolysis by streptococci is evidently hindered or decreased by the presence of glucose in the agar mediums; and, on the other hand, acid is produced in almost all strains in glucose fluid mediums. Looking at both phenomena together, we might be led to ask, if the hindrance of hemolysin-production in sugar mediums is caused by simultaneous acid-formations. Before entering directly into the subject the following preliminary observations were made, which have some bearing on the problem.

## HEMOLYSIN TEST OF CULTURE FLUID AFTER INOCULATION

The streptococci which were used in this experiment were obtained from inflamed tonsils (1, 2, 3), erysipelas (4), and scarlatinal tonsils (5). They were cultivated, first, on the standard blood-agar plates; according to Becker,<sup>1</sup> distinctly hemolytic strains were selected and transplanted to ascites-broth (1:1) or horse-serum broth (1:1). These tubes were incubated for 8-15 hours at 37 C., then centrifugated, and the supernatant fluid used for the hemolysin test.

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TABLE 1  
SCHEME A FOR HEMOLYSIN TEST

Number of Tube	Quantity of Culture Fluid, C.c.	Physiologic Salt Solution, C.c.	Defibrinated Isotonic Human Blood Suspension, C.c.	Percentage of Culture Mediums	Percentage of Blood
1	0.1	4.70	0.2	2	4
2	0.25	4.55	0.20	5	4
3	0.50	4.30	0.20	10	4
4	0.75	4.05	0.20	15	4
5	1.00	3.80	0.20	20	4
6	1.50	3.30	0.20	30	4
7	2.00	2.80	0.20	40	4
8	2.50	2.20	0.20	50	4
9	3.00	1.80	0.20	60	4
10	3.50	1.30	0.20	70	4
11	4.00	0.80	0.20	80	4
12	4.50	0.30	0.20	90	4
13	4.80	....	0.20	96	4

The comparison of the hemolysin-production of the 2 kinds of broth mixtures will be discussed later. The necessary quantity of fluid for this test was determined. The mixtures were placed at 37 C. for 2 hours, in the ice-box for 12 hours, and the result recorded. Five strains of hemolytic streptococci were examined by this method.

TABLE 2  
THE HEMOLYTIC POWER OF THE STRAINS OF STREPTOCOCCI USED

No. of Strains	0.1 C.c.	0.25	0.5	0.75	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	4.8
1	—	—	—	+	+	+	+	+	+	+	+	+	+
2	—	—	—	+	+	+	+	+	+	+	+	+	+
3	—	±	±	+	+	+	+	+	+	+	+	+	+
4	—	±	+	+	+	+	+	+	+	+	+	+	+
5	—	±	+	+	+	+	+	+	+	+	+	+	+

After this a mixture of 0.5 c.c. and 1 c.c. of broth was used to test the hemolysis with different quantities of blood.

TABLE 3  
SCHEME B<sub>1</sub> AND B<sub>2</sub> FOR HEMOLYSIN TEST

Scheme	Quantity of Culture Fluid, C.c.	Defibrinated Washed Human Blood, C.c.	Physiologic Salt Solution, C.c.	Percentage of Contained Blood
B <sub>1</sub>	0.5	0.05	4.45	1
	0.5	0.10	4.40	2
	0.5	0.15	4.35	3
	0.5	0.20	4.30	4
	0.5	0.25	4.25	5
	0.5	0.30	4.20	6
	0.5	0.35	4.15	7
	0.5	0.40	4.10	8
B <sub>2</sub>	1	0.05	3.95	1
	1	0.10	3.90	2
	1	0.15	3.85	3
	1	0.20	3.80	4
	1	0.25	3.75	5
	1	0.30	3.70	6
	1	0.35	3.65	7
	1	0.40	3.60	8

TABLE 4  
HEMOLYSIN TESTS WITH DIFFERENT PERCENTAGES OF BLOOD \*

Number of Strain	Percentage of Blood							
	1%	2%	3%	4%	5%	6%	7%	8%
1	$\pm$ +	+	+	+	$\pm$ +	$\mp$ +	$\mp$ +	$\mp$ +
2	$\pm$ +	+	+	+	$\mp$ +	$\mp$ +	$\mp$ +	$\mp$ +
3	$\pm$ +	$\pm$ +	+	+	— +	$\mp$ +	$\mp$ +	— +
4	+	+	+	+	+	+	+	+
5	$\pm$ $\pm$	+	+	+	$\mp$ +	$\mp$ +	— +	— +

\* In the tables the mark ( $\pm$ ) signifies ambiguous result and the marks ( $\mp$ ) indicates incomplete hemolysis, that is, some unhemolyzed corpuscles remained at bottom.

Considering that the variations of hemolytic power may be easily noted at the border of positivity, I used 1 c.c. of the culture fluid, with 4% blood in the subsequent examinations, because if a larger amount of blood is used the slight decrease of hemolytic power might in some cases fail to attract attention.

*Sensitivity of Erythrocytes of Different Animals to Hemolysin.*—The blood of human beings, dogs, goats, sheep, rabbits, and guinea-pigs was used. The samples of blood were taken under sterile conditions, and the red corpuscles washed 3 times with physiologic salt solution, centrifugated, and mixed with salt solution equal in quantity to the serum taken off. The test was made according to Table 1.

TABLE 5  
HEMOLYSIN TESTS WITH THE BLOOD OF DIFFERENT SPECIES

Number of Strain	Quantity of Culture Fluid, C.c.	Human Blood	Dog Blood	Goat Blood	Sheep Blood	Rabbit Blood	Guinea-pig Blood
3	0.1	—	—	—	—	—	—
	0.3	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.5	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.7	+	+	+	+	+	+
	1.0	+	+	+	+	+	+
4	0.1	—	—	—	—	—	—
	0.3	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.5	+	+	+	+	+	+
	0.7	+	+	+	+	+	+
	1.0	+	+	+	+	+	+
5	0.1	—	—	—	—	—	—
	0.3	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.5	+	+	+	+	+	+
	0.7	+	+	+	+	+	+
	1.0	+	+	+	+	+	+

Thus, in regard to sensitivity to hemolysin, the human blood corpuscle comes the first, and the corpuscles of guinea-pigs, rabbits, dogs, goats follow in the order given. The red corpuscle of sheep seems to have a slightly higher resistance to the hemolysin.

*Filtrability of Hemolysin.*—On the filtrability of hemolysin of streptococci the opinions of investigators have been divided; Some thought that the streptolysin was not filtrable; others claimed that it could be filtered. The recent publications on the subject record mostly positive results, although according to some authors (Hellens, etc.) the extent of filtrability varies.

In these experiments Berkefeld N filters and 15-hour cultures of streptococci in human or horse serum or ascites broth were used. The difference between the unfiltered culture and the filtrate with regard to hemolytic power is shown in Table 6.

TABLE 6  
COMPARISON OF HEMOLYTIC POWER OF UNFILTERED CULTURE FLUIDS

Number of Strain	Culture Fluids		0.1	0.25	0.5
2	Human serum + broth	Unfiltered	—	—	—
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	—	—
4		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	±
		Filtrate	—	—	—
5	Human serum + broth	Unfiltered	—	±	+
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	—	+
5		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	±
		Filtrate	—	—	—
5	Human serum + broth	Unfiltered	—	—	+
		Filtrate	—	—	—
	Horse serum + broth	Unfiltered	—	±	+
5		Filtrate	—	—	—
	Ascites fluid + broth	Unfiltered	—	—	+
		Filtrate	—	—	—

As shown, the filtrate in all cases undoubtedly possesses less hemolytic power than the unfiltered culture. Whether this difference is exclusively due to the living streptococci in the unfiltered culture is not easily decided, because in some cases the beginning of the hemolytic process in the unfiltered culture fluid could be observed even very soon after incubation, too soon indeed for any action of bacteria, while the filtrate did not cause any such phenomenon after a longer period.

For obtaining the hemolysin the human serum mixed with plain broth in equal parts seems to have a slight superiority over other mixtures. the ascites fluid and horse serum have about the same power for this purpose, yet in the case of Strain 4, the former showed an earlier production of the hemolysin than the latter, while the latter finally showed the greater production if cultivated for a longer period than the former. Moreover, we cannot overlook a certain predilection of certain strains of streptococci for particular mediums as regards hemolysin-formation.

*Time of Hemolysin-Production.*—Strongly hemolytic strains of streptococci sometimes clearly show the hemolytic halo on blood agar after 1 or 2 hours in the incubator (usually 5-8 hours), and the same results may be observed in fluid cultures. In most instances hemolysin begins to develop after 3 hours'

incubation at 37 C., and usually increases until the 15th-18th hour. Some strains do not show much difference in hemolytic power between 18 and 24 hours, while others show an evident reduction of hemolysin after 18 or 24 hours' cultivation. The streptolysin survives usually only a short time in fluid mediums. This has been noted previously by many. Although it is sometimes demonstrated 3-5 or 7 days after cultivation, this is rather exceptional, and generally the degree of hemolytic power is rapidly reduced after 24 hours. Even in the refrigerator the hemolysin shows a marked decrease hour by hour (Table 7).

The figures in Table 7 indicate the hemolytic power expressed by the reciprocal value of the culture fluids used.

TABLE 6—Continued  
COMPARISON OF HEMOLYTIC POWER OF UNFILTERED CULTURE FLUIDS

0.75	1.0	1.5	2.0	2.5	3.0	3.5	4.0
+	+	+	+	+	+	+	+
—	±	+	+	+	±	+	+
±	±	+	+	+	±	+	+
+	+	+	+	+	+	+	+
—	+	+	±	±	+	+	+
+	±	+	±	+	+	+	+
—	±	+	±	+	+	+	+
+	+	+	+	+	+	+	+
—	±	+	+	±	+	+	+
+	±	—	±	+	+	+	+
—	±	—	+	+	+	+	+
+	+	+	+	+	+	+	+
—	±	—	+	+	+	+	+
+	±	+	+	+	±	+	+
—	±	—	—	+	±	+	+
+	+	+	+	—	+	+	+
—	±	—	±	±	+	+	+

TABLE 7  
DURATION OF CULTIVATION IN RELATION TO PRODUCTION OF HEMOLYSIN

Number of Strain	Hours of Incubation										
	1	3	5	8	10	15	18	24	36	48	72
2	—	—	0.5	1	1	1	1	0.5	—	—	—
4	—	0.4	0.66	1	2	2	2	1.3	0.33	0.25	—
5	—	—	0.33	1	1	2	2	1	0.33	—	—

TABLE 8  
FERMENTATION TEST OF THE STRAIN OF STREPTOCOCCI USED

No. of Strain	Plain Broth	Glucose	Inulin	Lactose	Maltose	Mannite	Neutral Red	Raffinose	Saccharose	Saline
1	—	+	—	+	+	—	—	—	+	±
2	—	+	—	+	+	—	—	+	+	±
3	—	+	—	—	+	—	—	—	—	±
4	—	+	—	+	+	—	—	—	+	±
5	—	+	—	+	+	—	—	—	+	+

*Acid Formation by Streptococci.*—The strains of streptococci used in these experiments varied in fermentative power, as shown in Table 8.

To decide whether acid is produced or not, the culture mediums were titrated before and after cultivation, and the difference of acidity in both titrations was regarded as the degree of acid production.

*Time of Cultivation in Relation to Acid-Formation.*—Using 1% glucose broth for cultures, the results in Table 9 were obtained.

TABLE 9  
TIME OF CULTIVATION IN RELATION TO ACID-FORMATION

Number of Strain	Hours of Incubation										
	3	5	8	10	15	18	24	36	48	72	96
1	—	—	—	2.5	3.2	3.2	5.0	5.0	5.0	4.8	5.0
2	—	0.1	0.1	0.3	1.5	2.8	4.1	4.0	4.1	4.1	0
3	—	—	0.3	0.8	2.5	3.0	5.2	5.2	5.0	5.2	5.2
4	—	—	1.0	1.2	3.0	4.4	5.6	5.6	5.6	0	0
5	0.5	0.6	0.5	1.0	2.4	3.8	4.8	4.8	4.8	0	0

The acid-formation was not visible before 3 hours. Within 5-10 hours, when hemolysin-production seems usually to take place, the production of acid appeared to be very slight; after this, however, the degree of acid-formation was rapidly increased, and it reached the climax in 24-48 hours. After 48 hours the amount of acid produced was not increased, a fact which is not difficult to understand, since it is well known that streptococci usually cease to grow in fluid mediums after 48 hours.

*Percentage of Glucose Used in the Broth in Proportion to Acid-Formation.*—The percentage of glucose used in the broth is of some importance in the production of acid: 1-3% of glucose showed in many cases the same high formation of acid. In excess of 5% of glucose, acid-formation seemed to be markedly reduced, although the growth of streptococci was not distinctly hindered by the higher content of glucose (Table 10).

TABLE 10  
PERCENTAGE OF GLUCOSE IN PROPORTION TO ACID-FORMATION

No. of Strain	Medium	0.25	0.5%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	15%	20%	25%
2	Broth.....	2.0	4.0	4.0	4.0	3.8	3.2	2.5	—	—	—	—	—	—	—	—
	Broth + horse serum	2.8	3.5	4.1	4.1	4.1	3.8	3.2	3.2	1.0	0.8	—	0.2	—	—	—
	Broth + ascites fluid	2.5	3.8	4.2	4.1	4.0	4.1	3.8	3.0	0.8	0.8	—	0.1	—	—	—
4	Broth.....	2.0	2.4	5.4	5.4	5.0	3.4	3.5	—	—	2.0	1.8	0.5	—	—	—
	Broth + horse serum	3.0	5.2	5.6	5.6	5.6	3.9	5.2	4.0	4.2	2.8	3.0	—	—	—	—
	Broth + ascites fluid	2.8	5.0	5.5	5.3	5.5	4.8	5.0	3.5	3.0	—	1.0	—	—	—	—
5	Broth.....	3.0	4.5	4.8	4.8	4.2	3.8	2.8	3.0	2.8	2.0	1.8	—	—	—	—
	Broth + horse serum	3.5	4.8	4.8	4.8	4.5	4.3	4.0	4.0	3.8	2.0	2.3	1.8	0.5	—	—
	Broth + ascites fluid	3.2	4.8	4.8	4.8	4.3	4.1	4.5	4.1	1.8	1.8	1.5	0.5	—	—	—

If we take into consideration the difference between the 0.5% and 3% glucose broth, the 3 kinds of broth did not show much difference in acid-production by cultivation of streptococci. The fact that the broths with horse serum or ascites fluid indicated acid-formation even in a higher content of glucose than the broth without such mixture might be because they are more suitable mediums



for streptococci than the unmixed glucose broth, although the streptococci would grow little less in the latter than in the former. In other words, in the glucose broth to which horse serum or ascites fluid had been added the streptococci could exhibit and maintain their vitality in a higher content of glucose than in the simple glucose broth.

*Hemolysis Shown by the Acid Produced.*—The acid produced in glucose broth by streptococci was strongly hemolytic for the washed erythrocytes of all the animals examined.

The test was made as follows: The broth cultures of varying contents of glucose were incubated for 24 hours, and then heated to 60 C. for 30 minutes. Five c.c. of each broth culture of the different percentages of glucose were taken and neutralized with 1/20 normal sodium hydrate to the point of acidity of the original broth before incubation. Thus the degree of the acid produced was determined. The other portions were used in the hemolysin test in graduated quantities.

TABLE 11  
HEMOLYTIC POWER OF ACID PRODUCED

Number of Strain	Percentage of Glucose	Quantity of Culture Fluid, C.c.					Titer of Acid Produced
		0.1	0.3	0.5	0.7	1.0	
4	0.5	—	—	±	+	+	2.4
	1.0	—	+	+	+	+	5.4
	2	±	+	+	+	+	5.4
	3	—	±	+	+	+	5.0
	4	—	—	+	+	+	3.4
	5	—	—	+	+	+	3.5

The same test was made with Strains 2 and 5, the results being about the same.

*Hemolysin-Production in Glucose Broth.*—In this experiment I added 1/20 normal sodium hydrate to culture mediums with different amounts of glucose, in order to reduce the acidity to the acid degree of plain broth, and after cultivation for 15 hours the necessary amount of normal sodium hydrate was once more added to neutralize the acid produced. The hemolysin test was then tried, according to Table 1.

TABLE 12  
HEMOLYSIN-PRODUCTION IN GLUCOSE BROTH

Number of Strain	Plain Broth	Glucose Broth, %						
		0.25	0.5	1	2	3	4	5
1	+(0.75)	—	—	—	—	—	—	—
2	+(0.75)	+(2.5)	+(2.5)	—	—	—	—	—
3	+(0.75)	+(2.0)	+(2.0)	+(2.5)	—	—	—	—
4	+(0.75)	+(1.5)	+(1.5)	+(2.0)	+(2.0)	+(2.5)	—	—
5	+(0.5)	—	—	—	—	—	—	—

The figures within the brackets indicate the quantities of culture fluid necessary to give positive results in the hemolytic test.

I found in this test that distinct hemolysin-production occurred only in plain broth, seldom in a weak content of glucose, and very rarely in 3% of glucose as in the case of Strain 4, while the titration of acid produced in that same percentage in dicated 5.

*Longevity of Streptococci in Broth with Various Amounts of Glucose.*—Experiments have been made to ascertain what influence the various percentages

of glucose in broth may have on the power of growth of streptococci in relation to survival.

After 24 hours in the incubator, the tubes which contained the higher content of glucose (6 or 7%) scarcely showed turbidity at the bottom; however, the streptococci could be found in stained films of fluid at the bottom of the tubes.

The form and shape of the bacteria were not much changed, even in broth which contained 9 or 10% of glucose. With a higher percentage of glucose some of the streptococci were distinctly agglutinated, but the others still remained in the normal chain form.

Transplants on blood-agar slants after various periods at room temperature gave the results shown in Table 13.

TABLE 13  
SURVIVAL OF STREPTOCOCCI IN GLUCOSE BROTH OF DIFFERENT STRENGTHS

Number of Strain	Weeks	Plain Broth	Glucose Broth, %														
			0.25	0.5	1	2	3	4	5	6	7	8	9	10	15	20	25
2	1	—	+	+	+	+	+	+	+	—	+	+	—	+	+	—	—
	3	—	—	+	+	+	+	+	+	—	—	—	—	—	—	—	—
	5	—	—	+	+	+	+	—	—	—	—	—	—	—	—	—	—
	10	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—	—
4	1	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
	3	—	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
	5	—	—	+	+	—	+	—	—	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	1	+	+	+	+	+	—	+	+	+	—	+	+	—	+	—	—
	3	+	+	+	+	+	—	+	+	—	—	—	—	—	—	—	—
	5	—	—	+	—	—	—	—	+	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

As shown, the glucose content of 0.5-2% was most favorable for survival of streptococci. With an increased percentage of glucose the streptococci could live for a certain time only.

#### DISCUSSION

Hemolysin is found in 1% glucose broth cultures, while the content of acid produced in the same culture reaches a comparatively high amount. At all events, if the hemolysin-production is unmistakably reduced or totally hindered in fluid mediums containing glucose it does not seem to be true that the hemolysin-formation is checked or destroyed by the acid produced, because in some cases the hemolysin was still found even in a high degree of acid-formation; in other cases the former was not found at all when there was very slight or no formation of the acid in glucose broth. The probable explanation is that, although the hemolysin-production is an important characteristic for the classification of certain kinds of streptococci, it needs an adequate culture medium, and can be easily hindered by an inadequate amount of a certain ingredient of the mediums and not by the produced acid. To repeat, the hemolysin does not seem to appear at all in a medium

inadequate for its production regardless of whether the acid is produced or not.

The formation of hemolysin reaches its climax in 8-15 hours, and after about 18-24 hours is rather rapidly diminished in quantity; while the production of acid is usually slow in the first 5-10 hours, and most marked in 24-48 hours after incubation. The degree of acid-formation is highest between a 0.5 and 2 percentage of glucose content, and decreases in intensity above 5%. At any rate, if the content of glucose exceeds a certain limit, the power of acid-formation of streptococci is undoubtedly hindered, although the ability of streptococci to live and to grow is not yet completely checked by such a high content of glucose. Thus from my experiments it seems probable that the ability to produce hemolysin is an attribute which is not always concomitant with the vitality of streptococci, and that it is easily checked by the addition of a certain quantity of glucose. In comparison, the acid-formation goes hand in hand with the vitality of streptococci, although the parallel is not constant, that is, if in a high content of glucose the streptococci lose, more or less, the ability of acid-formation they may yet retain vitality without much change. •

Taking into consideration the results of all observations, the following conclusions seem warranted:

Hemolysin-production by streptococci in glucose-containing fluid mediums does not stand with regard to time in reciprocal relation to acid-formation.

The ability of streptococci to produce hemolysin in glucose-containing mediums is checked by the glucose, and can not be considered hindered or destroyed by simultaneous acid-formation.

The ability to form acid in its turn does not altogether coincide with the ability of streptococci to live. It can be reduced or totally hindered in a higher content of glucose in fluid mediums, although the ability of streptococci to live still remains in some degree.

The production of hemolysin begins and ends earlier than the production of acid. The former can be demonstrated in many cases after 3-5 hours of cultivation, and reaches its climax in 15-18 hours, while the latter seldom appears so early (that is, within 3-5 hours), and its climax occurs between 24-48 hours.

Although the ability to produce hemolysin and acid by streptococci is important for their differentiation, yet they are not in concordance with the power of streptococci to live. In other words, the vitality of streptococci can exist intact, even if the power to form hemolysin and acid is nullified in certain culture mediums.

## SOME FACTORS IN SWIMMING POOL CONTROL \*

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The increase in popularity of public swimming pools and their installation as part of the equipment in nearly all departments of physical education has made the sanitary control of these pools an important problem.

As Mannheimer,<sup>1</sup> Levine,<sup>2</sup> Lewis<sup>3</sup>, and others, have reviewed the literature adequately, it is not necessary to do so now. Mannheimer emphasizes the potential danger of swimming pools in the spread of disease.

For several years past it has devolved on the staff of the Wisconsin State Laboratory of Hygiene to conduct inspections and make recommendations for the control of the 2 pools at the University of Wisconsin. The pool for men has proved an especially interesting subject for investigation, both on account of its appointments and the large number of students who use it. This pool is located on the ground floor of the gymnasium, in the central part of the large locker room. On both sides of the shallow end of the pool are located the showers. The pool is not enclosed nor separated from the showers or lockers, but the contour is well above the general level of the floor of the room. The inside of the tank is lined, with the exception of a small square in the deep end, with white tile. The room is not well lighted. The water (Table 1) used in the pool is pumped directly from Lake Mendota and contains naturally much plankton life.

The results of 2 investigations of this pool constitute the material for this paper. The 1st was made to determine the effect of biweekly treatments with commercial chlorid of lime and copper sulphate, respectively, on the unfiltered lake water then in use; the 2nd and more extended investigation was made after the installation of a refiltration system.

### CHEMICAL DISINFECTION

The first set of observations was done in the fall of 1915, at which time record was made of the effect of using raw lake water, water treated biweekly

\* Received for publication June 19, 1917.

<sup>1</sup> Jour. Infect. Dis., 1914, 15, p. 159.

<sup>2</sup> Ibid., 1916, 18, p. 293.

<sup>3</sup> Jour. of Indust. and Engin. Chem., 1916, 8, p. 914.

with copper sulfate, 0.5 part per million equivalent of the anhydrous salt, and commercial chlorid of lime equivalent to 0.5 part per million available chlorin, respectively. The results of this work are given in Chart 1.

TABLE 1

COMPOSITION OF THE WATER OF LAKE MENDOTA (ANALYSIS IN PARTS PER MILLION)

(a) Mineral Analysis <sup>5</sup>	
Silica .....	15.2
Aluminum and Iron Oxids .....	2.2
Calcium .....	19.8
Magnesium .....	21.6
Sodium .....	3.6
Potassium .....	2.2
Carbonate radicle .....	77.2
Sulphate radicle .....	15.3
Chlorine .....	3
(b) Sanitary Analysis	
Turbidity .....	0
Odor .....	0
Color .....	0
Nitrogen as free ammonia .....	0.044
Nitrogen as albuminoid ammonia .....	0.264
Nitrogen as nitrites .....	0.002
Nitrogen as nitrates .....	0.08
Hardness by soap method .....	185
Alkalinity by methyl orange .....	167
Oxygen consumed .....	3.3
Total solids .....	202

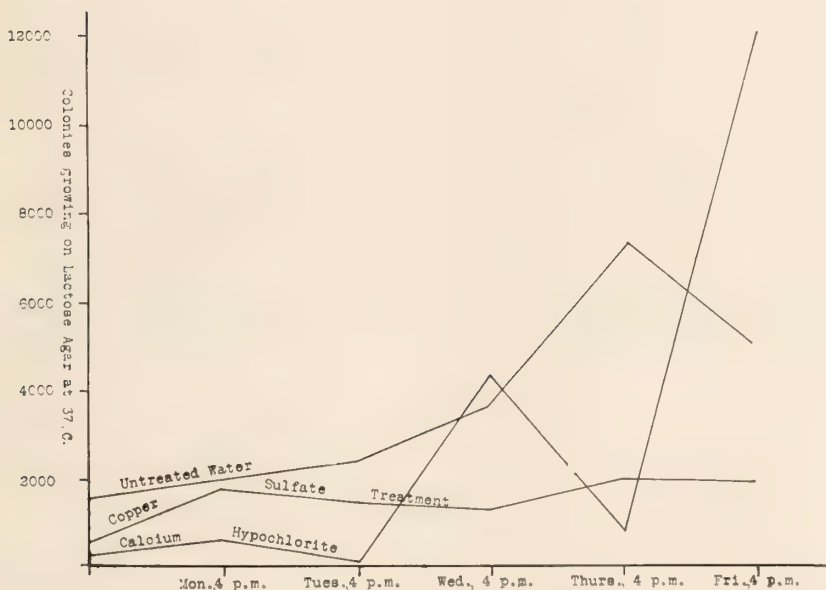


Chart 1. Effects of biweekly treatments

<sup>5</sup> The mineral analysis is taken from "The Underground and Surface Water Supply of Wisconsin," Wis. Sur. Bull., 35, p. 299.



It will be noted that while copper sulphate did not reduce the count as much as chlorid of lime, its effect was more lasting. In other words, it appears that the copper is not dissipated as readily as the 'bleach.' The immediate rise in the bacterial count directly after the hypochlorite is spent is probably due to the disintegration and oxidation products left available for bacterial food by the action of the hypochlorite on the organic matter originally present and accumulated.

#### REFILTRATION

In the late summer of 1916, a refiltration system was installed, and in the fall of that year it became necessary to conduct a series of observations to determine the best way to operate the system to obtain a satisfactory pool of water. A sketch of the system is shown in Figure 1.

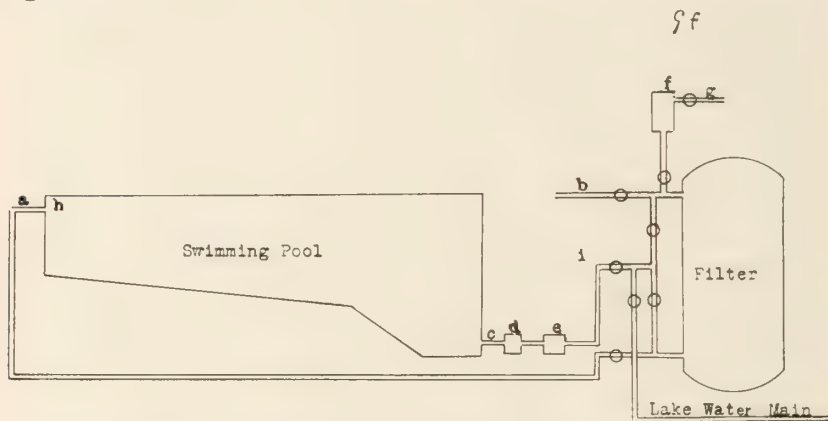


Fig. 1. Diagram of filter system (not drawn to scale). O = operating valves; a, inflow pipe to pool; b, pipe leading to sewer; c, outflow pipe to filter from pool; d, centrifugal pump; e, live steam heater; f, alum tank; g, city water main; h, place filtered sample taken; i, place unfiltered sample taken.

The problems were: Would refiltration alone under the conditions at this pool produce clear water and maintain a satisfactory bacterial count? Would it be necessary to use alum as a clarifier. Would it be necessary to treat the water in the tank with a disinfectant?

On Jan. 6, 1917, the tank was cleaned, filter washed, and tank refilled with filtered lake water without the use of alum. At noon, on January 8, the refiltration system was started and run 14 hours daily during the week. No alum or disinfectant was added during this week. The results are given in Table 2 (a).

TABLE 2  
REFILTRATION

(a) Results for week beginning Jan. 8, 1917.

Day of the Week	Number of Colonies Growing on Agar,* at 37 C.		
	Unfiltered	Filtered	Tank
Mon., p. m.....	5	7	13
Tues., p. m.....	4000	3000	2000
Wed., p. m.....	1000	850	800
Thurs., p. m.....	510	400	450
Fri., p. m.....	1575	865	1100

(b) Results for week beginning Jan. 15, 1917.

Mon., p. m.....	108	125	113
Tues., p. m.....	125	105	125
Wed., p. m.....	200	130	170
Thurs., p. m.....	250	165	170
Fri., p. m.....	100	65	70

(c) Results for week beginning Jan. 22, 1917.

Mon., p. m.....	110	80	135
Tues., p. m.....	80	40	60
Wed., p. m.....	60	26	55
Thurs., p. m.....	82	70	60
Fri., p. m.....	45	50	45

\* The agar used in this work was the standard litmus lactose agar.

During the next week, beginning January 15, the water from the week previous was left in the tank and treated with alum just before it entered the filter. No disinfectant was used but the refiltration system was operated 14 hours each day, as during the week before. The results are given in Table 2b. In the 3rd week, beginning January 22, both alum and commercial chlorid of lime were added each day. The latter was added directly to the pool in chlorid equivalent of 0.5 part per million. The results of this week are found in Table 2 (c). Table 2 (a) clearly shows that the filter was not satisfactorily removing the bacteria, and Table 2 (b) and (c) shows a low bacterial count in all samples, but a comparison of the unfiltered, filtered, and tank samples shows very little difference in the counts. The water in the tank was much clearer the last 2 weeks. During the 2nd and 3rd week of this series, alum in finely divided condition was detected in the tank sample, as well as in the other 2, filtered and unfiltered. The presence of alum in the tank water and the subsequent sedimentation caused thereby may in a measure explain the relatively few bacteria present in the water from the beginning of the 2nd week, for the filter showed no increase in efficiency. During the 2 weeks of alum treatment it was found necessary to remove the products of sedimentation with the vacuum cleaner 2 or 3 times a week in order to have a presentable tank of water.

The frequency which bacteria of the coli group were found to be present during these 3 weeks are given in Table 3.

TABLE 3  
FREQUENCY OF B. COLI DURING 3-WEEK PERIOD, JANUARY 8-29, INCLUSIVE

Amount Water, C.c.	First Week, %	Second Week, %	Third Week, %
10	93	60	66
1	21	6.6	6.6
0.1	0	0	0

During this test period of 3 weeks, chemical determinations were made for the content of nitrite and nitrate nitrogen and the chlorin as chlorids (Table 4).

TABLE 4  
CHEMICAL RESULTS

## (a) Results for week beginning Jan. 8, 1917

Day of the Week	Unfiltered			Filtered		
	NO <sub>2</sub>	NO <sub>3</sub>	Cl	NO <sub>2</sub>	NO <sub>3</sub>	Cl
Mon.....	0.001	0.1	2	0.001	0.08	2
Tues.....	0.002	....	2	0.002	....	2
Wed.....	0.003	0.08	2	0.003	0.08	2
Thurs.....	0.004	0.14	2.3	0.004	0.16	2.2
Fri.....	0.006	6.4	2.2	0.005	4	2

## (b) Results for week beginning Jan. 15, 1917

Mon.....	0.017	0.9	2	0.016	1.5	2.4
Tues.....	0.052	0.24	2.8	0.035	0.24	2.5
Wed.....	0.065	1.4	2.8	0.055	1.28	2.2
Thurs.....	0.104	0.4	2.5	0.122	0.24	2.5
Fri.....	0.208	2.63	2.8	0.208	1.85	2.8

## (c) Results for week beginning Jan. 22, 1917

Mon.....	0.63	0.14	2.5	0.63	0.14	2.5
Tues.....	0.8	0.18	3	0.8	0.18	3.1
Wed.....	0.9	0.14	3.6	0.9	0.14	3.5
Thurs.....	1	0.18	4	1	0.18	4.2
Fri.....	1	0.2	5	1	0.24	5

It is seen that while there is some variation in the nitrogen as nitrates and the chlorin as chlorids, it appears that the only significant variation from the normal is the gradual rise in nitrite nitrogen. This we find gradually increasing 0.001-1 part per million. The methyl orange alkalinity showed a slight decrease during the last 2 weeks of the 3.

During the 3 weeks beginning Feb. 26, 1917, tests were made on the pool using copper sulphate as the disinfectant. During this series the water was changed each week and the filter was kept running about 14 hours each day. On Saturday, February 24, the tank was cleaned and the filter was washed, and the tank subsequently filled without the use of alum. On Tuesday and Thursday evening, at 8 p. m., copper sulphate crystals were added in the quantity of 0.5 part per million. The results of this week are found in Table 5 (a).

TABLE 5  
CHEMICAL RESULTS

## (a) Results for week beginning Feb. 26, 1917

Day of the Week	Number of Colonies Growing on Agar, at 37 C.		
	Unfiltered	Filtered	Tank
Mon., p. m.....	300	1000	800
Tues., p. m.....	820	1500	480
Wed., p. m.....	170	35	3500
Thurs., p. m.....	75	5	950
Fri., p. m.....	175	....	175

## (b) Results for week beginning Mar. 5, 1917

Mon., p. m.....	1200	40	160
Tues., p. m.....	2500	1900	770
Wed., p. m.....	1200	205	950
Thurs., p. m.....	900	450	750
Fri., p. m.....	300	1000	410

## (c) Results for week beginning Mar. 12, 1917

Mon., p. m.....	3000	....	4500
Tues., p. m.....	1700	3100	1300
Wed., p. m.....	2500	525	1270
Thurs., p. m.....	2400	175	300
Fri., p. m.....	800	550	750

On Saturday, March 3, the tank was again emptied and cleaned, the filter washed, and the tank filled without the use of alum. Beginning Monday, March 5, there was added each day 0.5 part per million of copper sulphate crystals. The results of the week are given in Table 5 (b). Again on Satur-

day, March 10, the tank was prepared, the filter washed, and the tank filled without the use of alum. On each day during this week, copper sulphate crystals equivalent to 0.5 part per million of the anhydrous copper sulphate were added directly to the pool, as before. The results of this week are shown in Table 5 (c). The total amount of copper sulphate crystals entering the pool during each of these 3 weeks is:

First week.....	314 gm.
Second week.....	1100 gm.
Third week.....	1720 gm.

Note the variation in the count as shown in these tables under the title of 'tank'. During the 1st week, that of the biweekly treatment with copper sulphate, the high count was in the middle of the week and was about  $4\frac{1}{2}$  times the initial count on Monday. During the 2nd week, the high count was again in the middle of the week, and about 6 times the initial count on Monday. Contrasting these 2 results with that of the 3rd week—daily treatment with 0.5 part per million equivalent of anhydrous copper sulphate—the high count was here found in the beginning of the week, and was about  $3\frac{1}{2}$  times as large as on any other day. This appears to show that copper sulphate, when present in sufficient quantity, produces a satisfactory reduction in the bacterial count.

During the week beginning March 5, complete sanitary chemical determinations were made. The results are given in Table 6.

TABLE 6  
SANITARY CHEMICAL RESULTS ANALYSIS IN PARTS PER MILLION

	Mon.	Tues.	Wed.	Thurs.	Fri.
Turbidity .....	0	0	0	0	0
Odor .....	0	0	0	0	0
Color .....	0	0	0	0	0
Nitrogen as free ammonia.....	0.044	0.152	0.140	0.252	0.216
Nitrogen as albuminoid ammonia....	0.264	0.292	0.268	0.282	0.695
Nitrogen as nitrites.....	0.002	0.006	0.007	0.010	0.010
Nitrogen as nitrates.....	0.08	0.10	0.40	0.08	0.10
Chlorin as chlorides.....	1.5	2	2.5	2.2	2.5
Alkalinity as $\text{CaCO}_3$ .....	167	165	163	155	161
Total solids .....	202	206	214	230	214
Oxygen consumed .....	3.3	3.3	4.4	4.3	4.6

Table 6 shows that the nitrogen as ammonias gradually increases from 0.3 to about 0.9 part per million, and that the nitrites are also found to increase, as shown in Table 5.

On March 17, a few supplementary experiments were undertaken, the first of which was to determine the effect of sterilization of the filter in the evening, after it had been running 14 hours. On March 19, at 6 p. m., the filter was washed, and 7 parts per million of chlorin as commercial chlorid of lime were added to the water in the filter and allowed to stand over night. A similar sterilization was conducted on March 20. On Wednesday and Thursday evenings, March 21 and 22, similar experiments were made, except that 9 parts per million of copper sulphate crystals were used. The results are shown in Table 7.

On Wednesday and Thursday morning, after the treatment with hypochlorite and copper sulphate, respectively, it is seen that the condition of the water as it leaves the filter is satisfactory. While it is difficult to see just why the 1st treatment with hypochlorite on Monday, the 19th, and the

1st treatment with copper sulphate on Wednesday did not produce as good results, it appears that such sterilization is worthy of application, especially if the filter has been standing idle for some time.

TABLE 7  
EFFECT OF STERILIZATION OF FILTER

Day of the Week	Number of Colonies per C.c., Growing on Agar, at 37 C.	
	Unfiltered	Filtered
Mon., p. m.....	1050	9000
Tues., a. m.....	5500	2750
Tues., p. m.....	9000	900
Wed., a. m.....	10	0
Wed., p. m.....	110	235
Thurs., a. m.....	2500	2000
Thurs., p. m.....	2200	1250
Fri., a. m.....	7000	25
Fri., p. m.....	1250	200

On Saturday, March 31, the tank was prepared and the filter cleaned, as in previous experiments. The plan for this week was to start with a tank of filtered water and to note the effect of disinfection without refiltration. This corresponds to the treatment as shown in Chart 1, with the exception that in this latter test filtered lake water was used, while in the former unfiltered lake water was used. Accordingly, the tank was slowly filled from Saturday, March 31, to Monday, April 2, with the use of alum. At 8 p. m. on Tuesday, the pool was treated with 0.7 part per million chlorin as hypochlorite, and a similar treatment on Friday morning, at 8 o'clock. The results are shown in Table 8.

TABLE 8  
EFFECT OF DISINFECTION WITHOUT REFILTRATION

Week beginning April 2, 1917		Number of Colonies per Cc., Growing on Agar, at 37 C.		
Day of the Week		Shallow	Deep	Average
Mon., a. m.....		60	60	60
Mon., p. m.....		175	220	198
Tues., p. m.....		2000	1700	1850
Wed., p. m.....		450	220	335
Thurs., p. m.....		3000	2250	2625
Fri., p. m.....		25	9	17

These results are interesting when compared with the results as shown in Chart 1.

#### STERILIZATION OF THE FILTER

It appears possible that the filter may act in a manner not altogether unlike a septic tank, inasmuch as conditions are favorable for such action, when it is allowed to stand idle several hours a day. The first indications which we noticed of such action was the stench which accompanied the water from the washings of the filter after the filter had not been used for a few days, and that frequently the filtered sample contained more bacteria than the unfiltered or tank samples. On one of these occasions the filter was washed in the usual manner, and then chlorin as hypochlorite was added to the strength of about



7 parts per million, directly on to the filter. Samples were taken of the untreated and treated water and the counts made. The results are given in Table 9. It is seen that the action of the chlorin is very rapid, probably instantaneous, as the time factor recorded is evidently largely dependent on the mechanical factor of mixing, which in the case of the stationary filter is obviously slow.

TABLE 9  
EFFECT OF TREATING FILTER WITH HYPOCHLORITE

	1 C.c. on Agar, at 37 C.	1 Cc. on Gelatin, at 20 C.
Before treating .....	2500	30000
½ min. after treating.....	35	3750
3 min. after treating.....	10	1250
10 min. after treating.....	20	325
30 min. after treating.....	0	0

The first sample was taken from the wash water at beginning of the washings.

#### EFFICIENCY OF THE FILTER

From Tables 2 and 5 an idea may be obtained concerning the efficiency of the filter. In no instance does the difference in the bacterial counts in the unfiltered, filtered, and tank samples indicate that the filter alone can be relied on to satisfactorily remove bacteria, and that in some instances the filter seems to actually pollute the water. This latter is noted particularly after the filter has been washed, or after it has stood idle for some time without being washed.

If the efficiency is calculated by a comparison of the total bacteria in all of the unfiltered and filtered samples, then it is found to be 28.9% efficient. The efficiency is found to be 25% when calculated from the frequency with which bacteria of the coli group are found, using 40 10 c.c. samples for the calculation. These calculations, however, are obviously misleading, because at times the filter shows a negative efficiency. It does not constantly produce these percentages of efficiency, and, therefore, can not be relied on.

Tables 2 and 5 further seem to show that the use of alum has by some means reduced the bacterial counts in all of the samples. It was used by adding it to the water just as the water went on to the filter, hoping that the formation of aluminum hydroxid from the alkali and alkali earth carbonate in solution would clog the filter sufficiently to produce a material reduction in the bacterial count of the effluent. It did not do this. The water in the tank, however, was much clearer. This result is probably explained by the fact that the alum either passed through the filter in a finely divided state, or in solution afterwards to form the hydroxid, and precipitated out in the tank during the night,

carrying down much dirt and many bacteria. Such a conclusion is certainly substantiated by finding finely divided aluminum hydroxid in the water in the tank, the low bacterial counts, Table 2 (b) and (c), in all the samples, unfiltered, filtered, and tank, and by the presence every morning of much dirt on the bottom of the pool.

#### CONCLUSIONS

It appears from the studies of this pool that refiltration alone cannot be depended on to deliver a satisfactory tank of water. The initial filtration of the water appears to be of decided value in that it removes some of the suspended matter present in the raw lake water.

If refiltration is to be used it appears advisable to operate the filter continuously for 24 hours. If there is to be an idle period of several hours, the filter should be washed before the pump is stopped, and the filter treated with some disinfectant.

In conjunction with refiltration, it appears necessary to treat the water in the tank with either chlorin or copper sulphate in order to insure at all times a safe pool of water.

From the frequency with which alum was found passing through the filter, it seems advisable to pump the water through a coagulation basin before it enters the filter. This will give time for reaction, flocculation, and coagulation in particles large enough to 'clog' the filter and raise the efficiency from the beginning.

A vacuum cleaner should be a part of the equipment of the swimming pool.

The point of safety for the bathers can be as well secured by 1 initial filtration and subsequent treatment with either chlorin or copper sulphate, using a freshly filtered tank of water every week, as with refiltration and treatment, using the same tank of water for several weeks.

# PREPARATION AND METHOD OF USING TOXIN-ANTI-TOXIN MIXTURES FOR ACTIVE IMMUNIZATION AGAINST DIPHTHERIA \*

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Inquiries are made frequently for a detailed account of the method of preparing the mixtures of toxin-antitoxin, which have been used by us in the active immunization against diphtheria. The following communication is intended to answer some of the questions.

In our earlier work we began with mixtures of toxin-antitoxin, which were slightly overneutralized. Later, neutralized mixtures were used and finally slightly toxic mixtures were found to give the most satisfactory results.

The substances used for preparing the toxin-antitoxin consist of a well ripened and carefully standardized toxin and a preparation of antitoxin the strength of which has been closely determined.

*The Toxin.*—It is of advantage to have on hand a supply of from 50-100 liters of diphtheria toxin which has been passed through a Berkefeld filter and allowed to ripen in the ice-box for 6-12 months. The toxin is conveniently stored in the ice-box in liter bottles. During the first 3 months of ripening, a considerable proportion of the toxin is changed into toxoids. The toxin shows less change in its potency during the later months, and finally reaches a point where it is quite stable. By using the toxin at this time for the preparation of the toxin-antitoxin mixture, further changes of any considerable extent in the strength of the mixture will be avoided. The mixture will remain stable and will show unimpaired immunizing qualities for at least 6 months.

It is important to use a strong toxin with an L+ dose of at least 0.4 c.c. Each cubic centimeter of such toxin will represent 2.5 L+ doses. If the toxin is stronger, the number of L+ doses per cubic centimeter will be larger and the immunizing value of the mixture greater. It is difficult, however, to obtain a toxin stronger than one with an L+ dose of less than 0.2 c.c. A mixture prepared with a toxin of such strength ( $L + = 0.2$  c.c.) would contain about 5 neutralized doses of toxin and represent a toxin-antitoxin preparation of maximum efficiency. It is, of course, possible to give a larger dose than 1 c.c. of a toxin-antitoxin mixture prepared from a weaker toxin, and thus administer a similar number of neutralized L+ doses. By increasing the volume of the dose, however, we also increase the amount of foreign protein injected, especially in the case of the diphtheria bacillus. This is the particular agent involved in producing, in susceptible persons, the more severe types of local and constitutional reactions. In using a strong, ripened toxin, with an L+ dose of 0.4 to 0.2 c.c., representing 2.5 to 5 L+ doses of toxin in each cubic centimeter, we can prepare a suitable mixture of

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toxin-antitoxin. Within these limits the mixture will be efficient for purposes of active immunization.

*The Antitoxin.*—It is advisable to set aside 100-200 c.c. of a concentrated preparation of antitoxin, the strength of which has been closely determined. With a sufficient supply of standardized toxin and antitoxin on hand, additional quantities of toxin-antitoxin may be readily and accurately prepared.

*The Toxin-Antitoxin.*—After determining the amount of antitoxin to be added to the liter of toxin, some of the concentrated antitoxin is diluted 1:10 with sterile physiologic sodium chlorid solution to facilitate the exact measurement of the necessary number of units of antitoxin. To prepare a slightly toxic mixture, in which the desired proportion of toxin is 85% of an L+ dose to each unit of antitoxin, the amount of antitoxin necessary to be added to 1 L+ dose of toxin will be 1.17 units. For example: a toxin with an L+ dose of 0.4 c.c. contains 2.5 L+ doses per cubic centimeter and will require  $2.5 \times 1.17$  or 2.9 units of antitoxin to each cubic centimeter, and 2900 units to the liter of toxin. Where a stronger toxin is used, proportionately more antitoxin will have to be added.

After the mixture of toxin and antitoxin is thoroughly shaken, it is allowed to stand for 2 hours before sterility and potency tests are made. The toxicity is carefully determined in 2 guinea-pigs, one of the animals receiving subcutaneously 1 c.c., the other one 5 c.c. of the toxin-antitoxin. The animal receiving the smaller dose should show a slight induration; the other one injected with the larger amount should show a moderate or marked local induration and late paralysis, but should not die acutely of diphtheria poisoning. If the animal dies before the 5th day, it indicates an excess of free toxin in the mixture, which has to be carefully neutralized. It is evident, therefore, that to facilitate the quick preparation of additional amounts of toxin-antitoxin of uniform strength, it is necessary to have on hand a sufficiently large supply of the same preparations of toxin and of antitoxin.

*Bottling and Labelling.*—If the preparation is sterile and of proper strength, it is then put up in 10 c.c. bottles, and kept cold in the ice-box. We have found it of advantage, for purposes of identification, to give each lot of toxin an alphabetical letter, to which is added a number to designate each liter of the toxin-antitoxin mixture.

*Relative Value of Various Mixtures of Toxin-Antitoxin.*—A toxin which is excessively overneutralized and contains to each L+ dose from 10 to 100 units or more of antitoxin, will produce an active immunity in only a small proportion of nonimmunes. We have found that children, who were exposed to diphtheria and were given for purposes of immediate protection 1000 units of antitoxin with the 1st or the 2nd dose of toxin-antitoxin, did not respond as well to active immunization as those who received the toxin-antitoxin alone. This is due to an overneutralization of the toxin, which greatly impairs its immunizing value. The diminution in the antitoxin response after the injection of an overneutralized toxin has also been shown in the case of horses. These animals show, as a rule a much better antitoxin production after the injection of a mixture of diphtheria toxin-antitoxin, which contains 1.5 units of antitoxin to each L+ dose of toxin, than after the injection of a mixture containing 6 units of antitoxin to each L+ of toxin. On the other hand, a mixture of toxin-antitoxin which is underneutralized or which is dangerous to use on account of the excess of free toxin.

Mixtures which are either slightly overneutralized, neutral, or slightly toxic are safe and will give rise to an active immunity. Of the three, the slightly toxic



mixture will show the better antitoxin response, without producing any more local or constitutional symptoms than the neutral or overneutralized mixtures.

Table I shows the amounts of toxin and of antitoxin, and the percentage in each mixture of the L+ dose of toxin to one unit of antitoxin.

*Dose and Method of Administration.*—The mixture, as above described, is used undiluted. The dose is 1 c.c. injected subcutaneously in the arm at the insertion of the deltoid. The dose is repeated at weekly intervals until 3 injections have been given. For children under 1 year, the dose is 0.5 c.c. In the younger children, the local and constitutional symptoms following the injections of toxin-antitoxin are much less marked than in older children and adults. The difference is due to a greater susceptibility of older persons to the autolyzed protein of the diphtheria bacillus, which is present in the mixture of toxin-antitoxin.

*Determination of Immunity Response.*—The development of an active immunity is determined with the Schick test at the end of 3 months. The Schick retest is delayed for this period of time, so as to include all persons who will become actively immune. The results of some previous work carried out on numerous groups of persons have shown that the development of antitoxin in many persons is often a slow process, requiring 8-10 weeks before a sufficient amount is produced to inhibit the Schick test. The number of successfully

TABLE I  
MIXTURES OF DIPHTHERIA TOXIN-ANTITOXIN

Type Mixture	Amount of Toxin	Amount of Antitoxin in Units	Percent, L+ to 1 Unit of Antitoxin
1. Slightly toxic .....	1 L +	1.125—1.25	80-90
2. Neutral .....	1 L +	1.25 —1.5	65-80
3. Slightly overneutralized	1 L +	1.5 —2.0	50-65

immunized persons who finally show a negative Schick retest after 3 injections of toxin-antitoxin is 90-95%. After only 1 or 2 injections of toxin-antitoxin, a smaller number show the development of an active immunity: after 1 injection, about 60%; after 2 injections, about 80%. By reinjecting those who still give a positive Schick test, at the end of 3 months, with 2 or 3 more doses of toxin-antitoxin, an active immunity may be developed in almost all susceptible persons. In using the Schick test as an index of immunity, it is important not to mistake a pseudoreaction for a persistent positive Schick test.

The indications for active immunization with toxin-antitoxin may be divided into 2 groups: (a) as a general prophylactic measure, and (b) to control outbreaks of diphtheria.

The most suitable age period for prophylactic immunization is between 6 and 18 months. At this time of life nearly all children are susceptible to diphtheria. While only 15% give a positive Schick test at birth, almost all the remaining children lose their maternal immunity after the 6th to 9th month of life and then show a positive Schick reaction. It is, therefore, a safe and convenient rule to follow to



immunize with toxin-antitoxin all children below 18 months of age irrespective of the Schick test they may show at the time of immunization.

There are several advantages in this procedure. First, by omitting the Schick test in this group of children, the immunization will be greatly simplified and more readily applied by the large majority of general practitioners. Secondly, the local reaction after the toxin-antitoxin in young children is very slight or absent. Third, the morbidity and mortality from diphtheria is greatest between 1 and 4 years of age; therefore an early immunization on a large scale of young children is of great importance in controlling the disease. Fourth, the immunity which develops after injections of toxin-antitoxin is an active immunity, that persists for several years and possibly for life.

After the age of 18 months it is of advantage to apply the Schick test first, so as to exclude from immunization those children who show a negative reaction and are probably permanently immune. Young children can be reached in the homes, in infant asylums, in the milk stations, and in day nurseries. The children of the next age period are included in the preschool and school groups. These children may be reached in the public schools, in orphan asylums, and in the various other institutions. Among adults, those who come in contact with diphtheria and are constantly exposed and in danger should also be tested and immunized with toxin-antitoxin, if found to give a positive Schick reaction. Included in this group are especially physicians, nurses, and hospital attendants in contagious-disease hospitals.

The immunity developing from toxin-antitoxin is slow in appearing, and is, therefore, not a reliable safeguard in hospital wards where children are crowded together, and suffering from various contagious diseases. But in institutions where small outbreaks of diphtheria have occurred, or where diphtheria is more or less constantly present and clinical cases and bacillus carriers steadily appear, the use of antitoxin alone has often been insufficient to stamp out the disease, and the combined application of the Schick test and active immunization with toxin-antitoxin has given most successful and encouraging results.

## EFFECT OF ANILIN IN RABBITS\*

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The effect of anilin on human beings has been noted in a limited number of cases of industrial anilin poisoning. Malden,<sup>1</sup> in 1907, made a study of the blood of 13 men poisoned by anilin, working in the dye industry; Krause<sup>2</sup> described 2 cases; Trespe,<sup>3</sup> 2 cases; and more recently Luce and Hamilton<sup>4</sup> have reported cases in the dye and rubber industry, and Hudson<sup>5</sup> in the manufacture of explosives. Price, Jones, and Boycott<sup>6</sup> studied the blood and marrow of rabbits, in experimental anilin poisoning. It was thought interesting, during the course of an experiment on the effect of anilin on the production of antibodies in rabbits, to follow from day to day the changes in the blood count, to study the blood picture in stained smears, and also the sections of different tissues after death.

The anilin used was as pure as it was possible to make it, and was injected subcutaneously without being dissolved or diluted in anything. The leucocyte count was unaffected by the injection of anilin. In 1 set of 7 rabbits, injected with 0.2 c.c. on 4 successive days, whose leukocytes were counted every other day, the count never varied more than 3000 cells from what it was before the injections were begun. Another set of older rabbits which received larger doses, 0.8 c.c. on 3 successive days, gave the same results, although the red count was much reduced—in 1 case to 550,000. These results confirm the findings of Hudson but not those of Malden, who found a leukocytosis in 8 of 13 of his human cases.

Red counts were made every other day in the set of rabbits injected with 0.2 c.c. on 4 successive days. There was a rapid diminution in the red cells: 0.2 c.c. produced no appreciable reduction, 0.4 c.c., a reduction of about 1 million per c.c., and 0.8 c.c., a reduction of 2 or

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<sup>1</sup> Jour. Hyg., 1907, 7, p. 672.

<sup>2</sup> Med. Klin., 1908, 55, p. 10.

<sup>3</sup> München. med. Wchnschr., 1911, 58, p. 1720.

<sup>4</sup> Jour. Am. Med. Assn., 1916, 66, p. 1441.

<sup>5</sup> Med. Rec., 1917, 91, p. 89.

<sup>6</sup> Guy's Hosp. Rep., 1909, 63, p. 309.

3 million. In the animals that survived for any time after the injections were stopped, there was a gradual return toward normal. In one case, on the 8th day after the last injection, the count had risen from 1,970,000 to 2,860,000; in another from 1,310,000 to 2,670,000. None of the animals survived long enough to show how much time a complete recovery would take.

The differential counts failed to vary strikingly from normal. There was a slight increase in the polymorphonuclear leukocytes during the course of the injections in every case, but in none was it more than a 7% increase above the normal taken from the counts before the injections began. The eosinophils and basophils showed no appreciable variation. These results are somewhat surprising in view of Malden's findings in human blood, where in 5 of 9 cases there was a reduction in the polymorphonuclear cells and an increase in the mononuclears.

The morphologic changes in the red cells were much more striking. In every case there was marked metachromasia. It began to appear after the injection of 0.4 c.c., and increased till the full dose of 1 c.c. had been given. It gradually disappeared after the injections were stopped, and was hardly noticeable on the 10th day after the last injection. Great variation in size followed closely the course of the metachromasia, the cells ranging from 3-10 microns, the larger ones being the more numerous. Most of the cells averaged a little over 7 microns, as compared with 6 microns in the normal. Nucleated reds were occasionally found, 1 or 2 in the course of a differential count. Every animal showed stippling of the red cells. In some cases there were 3 or 4 stippled cells in a field and in others on the same day, after the same dose, a careful search of many fields had to be made. It was never found after 0.2 c.c. had been given, and in only 1 of 5 cases after 0.4 c.c. It appeared in all after 0.8 c.c. and 1.0 c.c. Five days after the last injection it was found in only 1 of 7 cases.

The changes in the tissues were studied after small doses of 0.2 c.c. for each of 2 days, medium doses, that is 0.2 c.c., for 4 or 5 successive days, and large doses, 0.8 c.c., every other day till the animals died. The only macroscopic changes in any of these rabbits were in the liver and spleen. In some cases after small and medium doses, and in every case after large doses, the liver was deeply bile-stained and spotted with multiple gray areas the size of an ordinary pin head. The spleen was generally a deeper red than normal, sometimes enlarged, and in

1 animal, which had received 2.4 c.c. of anilin in all, the spleen was 3 or 4 times the normal size.

Sections were made of kidney, liver, spleen, bone marrow of the femur, in a few instances of the marrow of all the long bones, and of the brain and spinal cord at different levels. The kidney, brain, and cord showed no changes. The liver, in over 50% of the animals, showed microscopically small areas of focal necrosis with some round cell infiltration, an occasional giant cell, some karyorrhexis in the center of the area, and the whole area walled off by a new growth of connective tissue. This necrosis was seen after small, medium, and large doses, as early as the 3rd day after the injection, and as late as the 21st day. In a few cases, the liver cords were shrunken, and in about one third, there were degeneration and edema of the liver cells around the central vein, and slight fatty changes. In the spleen, in all cases, there was an increase in the number of pigment cells. In some cases, the spleen was engorged with blood. That the spleen may play an important part in the toxic effect of anilin is suggested by the resistance shown by an old splenectomized rabbit, which stood 6.4 c.c. of anilin given in doses of 0.8 c.c. in the course of 12 days, nearly 3 times as much as any other animal stood.

The bone marrow of the femur was studied carefully in sections and in smears. The smears were treated with alphanaphthol and pyronin, according to the method of Graham,<sup>7</sup> to discover whether the myelocytes of the marrow, the red cells, or the nongranular cells were more affected. The changes in the marrow were very marked. Injections of as small amounts as 0.2 c.c. were followed in as short a time as 2 days by a marked hyperplasia, which replaced nearly all the fat. Larger doses produce solidly cellular marrows, which continued fairly cellular until 9 or 10 days after the injections were stopped. The fat did not return to any extent when the hyperplasia passed away, and its place was taken by a homogeneous substance. From a study of the sections and smears, this hyperplasia seems due to an increase in the normoblasts and premyelocytes, or nongranular cells. The giant cells were not markedly increased and showed no particular phagocytosis of leukocytes, as do those in the marrow of toluene rabbits.<sup>8</sup> The kinetic figures were not noticeably increased.

As normal rabbits at necropsy may show lesions of the liver simulating those found after anilin injections, guinea-pigs which less com-

<sup>7</sup> Jour. Med. Research, 1916, 35, p. 231.

<sup>8</sup> Brown: Tr. Chicago Path. Soc., 1917, x, p. 184.



monly show such lesions, were injected subcutaneously with 0.1 c.c. of anilin for 4 days, and the red and white cells counted. As the red count was not affected by this dose, and the only reaction seemed to be a local induration of the skin and subsequent ulceration, the dose was increased to 0.2 c.c. for 2 days, then 0.4 c.c. for 2 days, and finally 0.6 c.c. for 2 days, when the animals died. In all, each of these 3 pigs received 2.8 c.c. of anilin. The ulcers at the site of inoculation became worse, but the red count was not affected, and the stippling, anisocytosis, and metachromasia were slight. At necropsy, one animal showed focal necrosis of the liver in every way like that found in half the rabbits. The other 2 showed no changes in the liver. The spleen in all 3 was filled with pigment cells.

To test further the part of the spleen in the toxic effect, 5 rabbits were splenectomized, and 10 days later rather large doses of anilin were given subcutaneously, 0.4 c.c. for 4 days, and then 0.8 c.c. for 3 or 4 days, till the animals died. These animals stood, on the average, about twice as much anilin, and the red count was reduced about half as rapidly as in the nonsplenectomized ones. The resistance of the erythrocytes of these animals to hypotonic salt solutions did not appear to be increased. At necropsy 4 of these 5 splenectomized rabbits showed degeneration of the protoplasm of the liver cells about the central vein, and the 5th, a focal necrosis of the liver with collections of round cells and giant cell-formation.

Many of the rabbits injected with anilin were injected also with sheep blood, and the course of the resulting antishoop lysin and precipitin determined. In all cases, 25 c.c. of sheep blood were injected intraperitoneally at one time. The results indicate that when anilin is injected subcutaneously in the quantities indicated and sheep blood intraperitoneally, at about the same time, there is no restraint of antibody-formation. In 1 series in which the anilin was injected intraperitoneally and the blood also intraperitoneally, but several days later, there was marked restraint of antibody-production. In this case, questions arise as to the possible effect conditions produced by anilin in the peritoneal cavity may have had on the blood and its absorption.

#### CONCLUSIONS

In rabbits, anilin has a specific affinity for erythrocytes, greatly diminishing the number in large doses, and in small doses causing signs of degeneration in stippling, metachromasia, and anisocytosis.



It has no effect on the leukocytes of the blood or the myelocytes of the marrow. It may cause areas of focal necrosis in the liver, and there is evidence of blood destruction in the spleen. The spleen appears to play some rôle in the toxic effect, splenectomized rabbits being more resistant and their red cells reduced less quickly than non-splenectomized rabbits. It brings about a transitory hyperplasia of the bone marrow due to an increase in the normoblasts and nongranular cells.

In rabbits, anilin does not appear to affect the production of antibodies, except possibly when both the anilin and the antigen are injected intraperitoneally.

In guinea-pigs, anilin causes a local induration and ulceration of the skin at the site of inoculation, but does not reduce the red count, although there is some evidence of blood destruction in increased pigmentation of the spleen; in this animal, also, anilin may cause focal necrosis of the liver.

As the effects of anilin in guinea-pigs is not quite the same as in rabbits, no direct conclusion may be drawn from these experiments as to its effects in man.

# WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM MORE THAN A YEAR OLD\*

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In a previous report<sup>1</sup> I showed that glycerol is a suitable preservative for human serum intended for Wassermann reaction. Human serum was preserved for 3 months without any noticeable change in the result. This report deals with serum that has been preserved with glycerol for more than a year. As most of the specimens were reported on in the previous article, I shall arrange the tests and tables in accordance with the plan adopted at that time.

The Wassermann method with human hemolytic serum was used in this investigation.

As complement, the pooled serums of 2 or 3 guinea-pigs were used in quantities of 0.05, 0.025, and 0.0125 c.c.

The antigen was alcoholic extract of human heart muscle, and, as test dose, the largest quantity that was not anticomplementary in the antigen control was used. An antigen control was used with each lot of serum tested.

As hemolytic amboceptor, the serum of rabbits immunized with washed human blood corpuscles was used in doses of 1 and 1.25 units per tube.

The term unit was applied to the smallest quantity of hemolytic serum which, with 0.025 c.c. of complement, dissolved 0.25 c.c. of a 2.5% suspension of washed blood corpuscles in 1 hour. The total quantity in each tube was made up to 1.25 c.c., the same proportions, but only half the quantities used in the tests previously reported. The mixture of diluted amboceptor and corpuscles was allowed to stand at room temperature for 30 minutes before the complement was added. After the complement had been added, and enough salt solution to each tube to bring the total quantity up to 1.25 c.c., the tubes were placed in the incubator at about 37 C., for 1 hour.

Human corpuscles from nonsyphilitic persons, well washed, were used in doses of 0.25 c.c. of a 2.5% suspension in physiologic salt solution. The corpuscles were sensitized for 30 minutes before they were added to the serum-complement-antigen mixture.

The technic used in this investigation differed slightly from that described in previous reports. Of serum, complement, and antigen, one half of the previous quantities were used; of corpuscles, only one fourth, and of hemolytic amboceptor, enough to dissolve this small test dose of corpuscles. Before testing, the human serums were heated to about 55.5 C., for 30 minutes, and 0.6 c.c. of serum-glycerol mixture was diluted to 1.5 c.c. with physiologic salt solution (0.9 gm. of pure sodium chlorid in 1 liter of distilled water). Six

\* Received for publication July 9, 1917.

<sup>1</sup> Philippine Jour. Sc., Sect. B, 1916, 11, p. 87.

tubes, 1, 2, and 3 as antigen tubes and 1', 2', and 3' as control tubes, were used in each test. Each tube received 0.25 c.c. of diluted serum. Of the 1st pair, Tubes 1 and 1', each received 0.25 c.c. of 1:5 dilution of complement serum; each of the 2nd pair, Tubes 2 and 2', received 0.25 c.c. of 1:10 dilution of complement serum; and each of the 3rd pair, Tubes 3 and 3', received 0.25 c.c. of 1:20 dilution of complement serum. Each of the antigen tubes, Tubes 1, 2, and 3, received a test dose of antigen; and the control tubes, Tubes 1', 2' and 3', received 0.25 c.c. of salt solution each. These mixtures were placed in the incubator at 37 C., for 1 hour. After having been in the incubator 1 hour, each tube received 0.5 c.c. of sensitized corpuscles, representing 0.25 c.c. of 2.5% suspension of washed corpuscles and 1 or 1¼ unit of hemolytic amboceptor. After shaking, the tubes were placed in the incubator at 37 C. for 1 hour; during this time and during the 30 minutes while the corpuscles were being sensitized, the mixture were repeatedly shaken to prevent the corpuscles from settling to the bottom. After having been in the incubator for 1 hour, the tubes were allowed to stand at room temperature for 2 hours, after which the results were read and recorded.

In the antigen control, 6 test tubes were used as in the test, but the human serum was omitted, and the volume was made up with physiologic salt solution. If there was no anticomplementary action in the antigen control, the dose of antigen was considered suitable. If there was anticomplementary action, the dose of antigen was decreased until it was no longer anticomplementary.

TEST 1.—The serum was drawn off the clot on the day after bleeding, and each serum was divided into 2 portions, A and B. Portion A was tested at once and Portion B, unheated, was mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered test tube. Portion B of each serum was tested on May 20, 1917. Immediately before testing the necessary quantity, 0.6 c.c., was heated to 56 C. for 30 minutes.

TABLE 1\*  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED IMMEDIATELY BEFORE TESTING

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Antigen Tubes			Control Tubes			
						1	2	3	1'	2'	3'	
4424	11/23/15	A	11/24/15	11/24/15	1	0	0	0	+	±	0	Strongly positive
		B	5/20/17	5/20/17	1.25	+	0	0	+	+	tr	Strongly positive
4425	11/23/15	A	11/24/15	11/24/15	1	+	tr	0	+	+	0	Moderately positive
		B	5/20/17	5/20/17	1.25	+	±	0	+	+	tr	Moderately positive
4427	11/23/15	A	11/24/15	11/24/15	1	0	0	0	+	±	0	Strongly positive
		B	5/20/17	5/20/17	1.25	+	0	0	+	+	tr	Strongly positive
4428	11/23/15	A	11/24/15	11/24/15	1	0	0	0	+	±	0	Strongly positive
		B	5/20/17	5/20/17	1.25	+	0	0	+	+	tr	Strongly positive
4429	11/23/15	A	11/24/15	11/24/15	1	0	0	0	+	+	0	Strongly positive
		B	5/20/17	5/20/17	1.25	±	0	0	+	+	tr	Strongly positive
4430	11/24/15	A	11/25/15	11/25/15	1	+	±	0	+	+	0	Weakly positive
		B	5/20/17	5/20/17	1.25	+	+	0	+	+	tr	Weakly positive
4432	11/24/15	A	11/25/15	11/25/15	1	0	0	0	+	±	0	Strongly positive
		B	5/20/17	5/20/17	1.25	±	0	0	+	+	tr	Strongly positive
4434	11/26/15	A	11/27/15	11/27/15	1	tr	0	0	+	±	0	Strongly positive
		B	5/20/17	5/20/17	1.25	+	0	0	+	+	0	Strongly positive

\* In all the tables + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis less than 50%; 0, no hemolysis.

TABLE 2

WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED BEFORE BEING MIXED WITH GLYCEROL

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Anti-gen Tubes			Control Tubes			
						1	2	3	1'	2'	3'	
4437	11/26/15	A	11/27/15	11/27/15	1	0	0	0	+	±	0	Strongly positive
		B	11/27/15	5/20/17	1.25	+	0	0	+	+	±	Strongly positive
4438	11/26/15	A	11/27/15	11/27/15	1	+	0	0	+	+	tr	Strongly positive
		B	11/27/15	5/20/17	1.25	+	tr	0	+	+	±	Strongly positive
4439	11/26/15	A	11/27/15	11/27/15	1	+	±	0	+	+	0	Weakly positive
		B	11/27/15	5/20/17	1.25	+	+	tr	+	+	±	Weakly positive
4440	11/27/15	A	11/28/15	11/28/15	1	tr	0	0	+	+	tr	Strongly positive
		B	11/28/15	5/20/17	1.25	±	0	0	+	+	tr	Strongly positive
4441	11/27/15	A	11/28/15	11/28/15	1	0	0	0	+	+	tr	Strongly positive
		B	11/28/15	5/20/17	1.25	tr	0	0	+	+	tr	Strongly positive
4442	11/27/15	A	11/28/15	11/28/15	1	+	0	0	+	+	tr	Strongly positive
		B	11/28/15	5/20/17	1.25	+	±	0	+	+	±	Strongly positive
4443	11/27/15	A	11/28/15	11/28/15	1	tr	0	0	+	+	0	Strongly positive
		B	11/28/15	5/20/17	1.25	+	±	0	+	+	±	Strongly positive
4444	11/27/15	A	11/28/15	11/28/15	1	+	tr	0	+	±	0	Weakly positive
		B	11/28/15	5/20/17	1.25	+	+	tr	+	+	±	Weakly positive

TABLE 3

WASSERMANN REACTION WITH HUMAN SERUM, HEATED BEFORE BEING MIXED WITH GLYCEROL

Number	Date of Bleeding	Portions (A, Non-glycero- lated; B, Glycer- olated)	Heated	Tested	Ambo- ceptor Unit	Readings					Result	
						Anti- gen Tubes		Con- trol Tubes				
						1	2	3	1'	2'		3'
4446	11/30/15	A	12/1/15	12/ 1/15	1	+	0	0	+	+	tr	Strongly positive
		B	12/1/15	5/26/17	1.25	+	tr	0	+	+	tr	Strongly positive
4447	11/30/15	A	12/1/15	12/ 1/15	1	0	0	0	+	+	0	Strongly positive
		B	12/1/15	5/26/17	1.25	0	0	0	+	+	0	Strongly positive
4448	11/30/15	A	12/1/15	12/ 1/15	1	+	tr	0	+	+	tr	Strongly positive
		B	12/1/15	5/26/17	1.25	+	tr	0	+	+	tr	Strongly positive
4449	11/30/15	A	12/1/15	12/ 1/15	1	0	0	0	+	+	tr	Strongly positive
		B	12/1/15	5/26/17	1.25	0	0	0	+	+	0	Strongly positive

TABLE 4

WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED IMMEDIATELY BEFORE TESTING

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Anti-gen Tubes			Control Tubes			
						1	2	3	1'	2'	3'	
4452	12/1/15	A	12/ 2/15	12/ 2/15	1	tr	0	0	+	+	tr	Strongly positive
		B	5/26/17	5/26/17	1.25	tr	0	0	+	+	0	Strongly positive
4453	12/1/15	A	12/ 2/15	12/ 2/15	1	+	0	0	+	±	0	Moderately positive
		B	5/26/17	5/26/17	1.25	0	0	0	tr	0	0	Anticomplementary
4454	12/1/15	A	12/ 2/15	12/ 2/15	1	tr	0	0	+	+	tr	Strongly positive
		B	5/26/17	5/26/17	1.25	+	0	0	+	+	tr	Strongly positive
4456	12/1/15	A	12/ 2/15	12/ 2/15	1	0	0	0	+	+	tr	Strongly positive
		B	5/26/17	5/26/17	1.25	0	0	0	+	±	0	Strongly positive

Table 1 shows that 8 serums gave practically the same results on May 20, 1917, as they did in November, 1915. None of them had become permanently anticomplementary.

TEST 2.—The serum was drawn off the clot the day after the bleeding, and each serum was divided into 2 portions, A and B. Portion A was tested immediately and Portion B was heated to about 55.5 C. for 30 minutes, mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered test tube. Without having been reheated, Portion B was tested on May 20, 1917.

Table 2 shows that the results obtained on May 20, 1917, were practically identical with the results obtained in November, 1915. The serums had not become anticomplementary and remained clear.

TEST 3.—The serum was drawn off the clots on the day after the bleeding, and each serum was divided into 2 portions, A and B. Portion A was tested the next day, and Portion B was heated to 55.5 C. for 30 minutes, and mixed with an equal volume of sterilized, chemically pure glycerol, placed in cold storage at about 7 C. until the latter part of April, 1916; then it was kept at room temperature in a cork-stoppered test tube.

Without having been reheated, Portion B was tested on May 26, 1917.

Table 3 shows that the results obtained were practically the same on May 26, 1917, as on Dec. 1, 1915. None of the serum had become anticomplementary or cloudy.

TEST 4.—The serum was drawn off the clot on the day after bleeding. Each serum was divided into 2 portions. Portion A was tested at once, and Portion B was mixed with an equal volume of sterilized, chemically pure glycerol, kept in the cold storage at about 7 C. until the latter part of April, 1916, then at room temperature. On May 26, 1917, the necessary quantity was heated to about 56 C. for 30 minutes and tested.

Table 4 shows that the results obtained on May 26, 1917, nearly correspond to those of Dec. 2, 1915. Serum 4453 had become anticomplementary.

TEST 5.—The serum was drawn off the clot on the day after bleeding. Each serum was divided into 2 portions. Portion A was tested at once, and Portion B was mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered tube. On May 27, 1917, the necessary quantity was heated to about 56 C. for 30 minutes and tested.

Table 5 shows that the results obtained with Serums 4535, 4536, 4537, and 4538 on May 27, 1917, were identical with the results obtained on Dec. 28, 1915. Serum 4538 had become moderately anticomplementary.

TEST 6.—The serum was drawn off the clot the day after bleeding. Each serum was divided into 2 portions, A and B. Portion A was tested at once, and Portion B was heated to about 55.5 C. for 30 minutes, mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered tube.

On May 27, 1917, Portion B was tested without having been reheated.

The results obtained on May 27, 1917, are identical with those obtained on Dec. 28, 1915. None of these serums had become anticomplementary.



TABLE 5  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED  
IMMEDIATELY BEFORE TESTING

Number	Date of Bleeding	Portions (A, Non-glycero- lated; B, Glycer- olated)	Heated	Tested	Ambo- ceptor Unit	Readings						Result
						Anti- gen Tubes			Con- trol Tubes			
						1	2	3	1'	2'	3'	
4535	12/27/15	A	12/28/15	12/28/15	1	++	tr	++	tr		Negative	
4536	12/27/15	B	5/27/17	5/27/17	1.25	++	0	++	0		Negative	
		A	12/28/15	12/28/15	1	++	tr	0	++	0	Negative	
4537	12/27/15	B	5/27/17	5/27/17	1.25	++	0	++	0		Negative	
		A	12/28/15	12/28/15	1	++	0	++	0		Negative	
4538	12/27/15	B	5/27/17	5/27/17	1.25	++	0	++	0		Negative	
		A	12/28/15	12/28/15	1	++	0	++	0		Negative	
		B	5/27/17	5/27/17	1.25	+	0	0	+	0	Negative	

TABLE 6  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED  
BEFORE BEING MIXED WITH GLYCEROL

Num- ber	Date of Bleeding	Portions (A, Non- glycero- lated; B, Glycer- olated)	Heated	Tested	Ambo- ceptor Unit	Readings						Result
						Anti- gen Tubes			Con- trol Tubes			
						1	2	3	1'	2'	3'	
4540	12/27/15	A	12/28/15	12/28/15	1	++	tr	++	tr		Negative	
		B	12/28/15	5/27/17	1.25	++	0	++	0		Negative	
4541	12/27/15	A	12/28/15	12/28/15	1	++	tr	++	tr		Negative	
		B	12/28/15	5/27/17	1.25	++	0	++	0		Negative	
4542	12/27/15	A	12/28/15	12/28/15	1	++	tr	++	tr		Negative	
		B	12/28/15	5/27/17	1.25	++	0	++	0		Negative	
4544	12/27/15	A	12/28/15	12/28/15	1	++	0	++	0		Negative	
		B	12/28/15	5/27/17	1.25	++	0	++	0		Negative	

TEST 7.—The serum was drawn off the clot on the day after bleeding. Each serum was divided into 2 portions, A and B. Portion A was tested immediately and Portion B, unheated, was mixed with an equal volume of sterilized, chemically pure glycerol, and kept in a cork-stoppered tube in cold storage until the latter part of April, 1916; then it was kept at room temperature.

On May 27, 1917, the necessary quantity was heated to 56 C. for 30 minutes and tested.

As Table 7 shows, 3 of the serums had become anticomplementary.

TEST 8.—The serums were drawn off the clot on the day after bleeding. Each serum was divided into 2 portions, A and B. Portion A was tested at once. Portion B was heated to about 55.5 C. for 30 minutes, mixed with an equal volume of sterilized, chemically pure glycerol, and kept in a cork-stoppered tube in cold storage at about 7 C. until the latter part of April, 1916; then it was kept at room temperature. On May 27, 1917, Portion B was tested.

The results, on May 27, 1917, are the same as on Dec. 30, 1915. No serum had become anticomplementary.

TEST 9.—About 24 hours after having been secured, the serum was drawn off the clot. Each serum was divided into 2 portions, A and B. Portion A was tested immediately. Portion B was heated to about 55.5 C. for 30 minutes, mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered test tube. On June 3, 1917, Portion B. was tested.

TABLE 7  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED  
IMMEDIATELY BEFORE TESTING

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Anti-gen Tubes			Con-trol Tubes			
						1	2	3	1'	2'	3'	
4546	12/29/15	A	12/30/15	12/30/15	1	+	+	tr	+	+	tr	Negative
		B	5/27/17	5/27/17	1.25	+	+	0	+	+	0	Negative
4548	12/29/15	A	12/30/15	12/30/15	1	+	+	tr	+	+	tr	Negative
		B	5/27/17	5/27/17	1.25	0	0	0	0	0	0	Anticomplementary
4549	12/29/15	A	12/30/15	12/30/15	1	+	+	tr	+	+	tr	Negative
		B	5/27/17	5/27/17	1.25	tr	0	0	tr	0	0	Anticomplementary
4550	12/29/15	A	12/30/15	12/30/15	1	+	+	tr	+	+	tr	Negative
		B	5/27/17	5/27/17	1.25	+	tr	0	+	tr	0	Negative

TABLE 8  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED  
BEFORE BEING MIXED WITH GLYCEROL

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Anti-gen Tubes			Control Tubes			
						1	2	3	1'	2'	3'	
4551	12/29/15	A	12/30/15	12/30/15	1	++	tr	+	+	tr	Negative	
4553	12/29/15	B	12/30/15	5/27/17	1.25	++	0	+	+	0	Negative	
		A	12/30/15	12/30/15	1	++	tr	+	+	tr	Negative	
4554	12/29/15	B	12/30/15	5/27/17	1.25	++	tr	+	+	tr	Negative	
		A	12/30/15	12/30/15	1	++	tr	+	+	tr	Negative	
4555	12/29/15	B	12/30/15	5/27/17	1.25	++	tr	+	+	tr	Negative	
		A	12/30/15	12/30/15	1	++	tr	+	+	tr	Negative	
		B	12/30/15	5/27/17	1.25	++	0	+	+	0	Negative	

TABLE 9  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED  
BEFORE BEING MIXED WITH GLYCEROL

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Tested	Amboceptor Unit	Readings						Result
						Anti-gen Tubes			Control Tubes			
						1	2	3	1'	2'	3'	
4662	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	+	+	+	+	Negative
		B	4/ 6/16	6/ 3/17	1.25	+	+	+	0	+	+	Negative
4664	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	+	+	+	+	Negative
		B	4/ 6/16	6/ 3/17	1.25	+	+	+	0	+	+	Negative
4667	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	+	+	+	+	Negative
		B	4/ 6/16	6/ 3/17	1.25	+	+	+	0	+	+	Negative
4669	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	+	+	+	+	Negative
		B	4/ 6/16	6/ 3/17	1.25	+	+	+	0	+	+	Negative
4673	4/ 7/16	A	4/ 8/16	4/ 8/16	1	+	+	+	+	+	+	Negative
		B	4/ 8/16	6/ 3/17	1.25	+	+	+	0	+	+	Negative
4684	4/ 9/16	A	4/10/16	4/10/16	1	+	+	+	+	+	+	Negative
		B	4/10/16	6/ 3/17	1.25	+	+	tr	+	tr	+	Negative
4650	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	tr	+	+	+	Weakly positive
		B	4/ 6/16	6/ 3/17	1.25	+	tr	0	+	+	+	Weakly positive
4652	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	tr	+	+	+	Weakly positive
		B	4/ 6/16	6/ 3/17	1.25	+	tr	0	+	+	+	Moderately positive
4707	4/11/16	A	4/12/16	4/12/16	1	+	+	tr	+	+	+	Weakly positive
		B	4/12/16	6/ 3/17	1.25	+	tr	0	+	+	+	Moderately positive
4657	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	0	+	+	+	Strongly positive
		B	4/ 6/16	6/ 3/17	1.25	+	0	0	+	+	+	Strongly positive
4668	4/ 5/16	A	4/ 6/16	4/ 6/16	1	+	+	0	+	+	+	Strongly positive
		B	4/ 6/16	6/ 3/17	1.25	+	0	0	+	+	+	Strongly positive
4653	4/ 5/16	A	4/ 6/16	4/ 6/16	1	0	0	0	+	+	+	Strongly positive
		B	4/ 6/16	6/ 3/17	1.25	0	0	0	+	+	0	Strongly positive

As Table 9 shows, the results obtained on June 3, 1917, were nearly identical with the results in April, 1916. Three serums, 4650, 4652, and 4707 reacted a little stronger than they did in April, 1916. None of the 12 serums had become anticomplementary.

TEST 10.—The serum was drawn off the clot about 24 hours after the bleeding. Each serum was divided into 2 portions, A and B. Portion A was tested immediately; Portion B was mixed with an equal volume of sterilized, chemically pure glycerol, and kept at room temperature in a cork-stoppered test tube. Seven days after having been mixed with glycerol, each serum was heated to about 55.5 C. for 30 minutes. After having been heated the serum was kept at room temperature.

On June 9, 1917, Portion B was tested before and after having been reheated.

Table 10 shows that the results obtained on June 9, 1917, were practically identical with the results in February, 1916. The serums had become slightly anticomplementary. The anticomplementary property was completely destroyed by heating to 56 C. for 30 minutes.

TABLE 10  
WASSERMANN REACTION WITH GLYCEROLATED HUMAN SERUM, HEATED 7 DAYS  
AFTER BEING MIXED WITH GLYCEROL

Number	Date of Bleeding	Portions (A, Non-glycerolated; B, Glycerolated)	Heated	Reheated	Tested	Amboceptor Unit	Readings						Result
							Anti-gen Tubes			Control Tubes			
							1	2	3	1'	2'	3'	
4618	2/25/16	A	2/26/16	6/ 9/17	2/26/16	1	+	+	tr	+	+	tr	Negative
		B	3/ 4/16		6/ 9/17	1.25	+	tr	0	+	tr	0	Negative
		B	3/ 4/16		6/ 9/17	1.25	+	+	tr	+	+	tr	Negative
4621	2/26/16	A	2/27/16		2/27/16	1	+	0	0	+	+	tr	Strongly positive
		B	3/ 5/16		6/ 9/17	1.25	+	0	0	+	+	0	Strongly positive
		B	3/ 5/16	6/ 9/17	6/ 9/17	1.25	+	0	0	+	+	tr	Strongly positive
4616	2/24/16	A	2/25/16		2/25/16	1	+	0	0	+	+	tr	Strongly positive
		B	3/ 3/16		6/ 9/17	1.25	tr	0	0	+	tr	0	Strongly positive
		B	3/ 3/16	6/ 9/17	6/ 9/17	1.25	+	0	0	+	+	tr	Strongly positive
4617	2/24/16	A	2/25/16		2/25/16	1	0	0	0	+	+	tr	Strongly positive
		B	3/ 3/16		6/ 9/17	1.25	0	0	0	+	±	0	Strongly positive
		B	3/ 3/16	6/ 9/17	6/ 9/17	1.25	±	0	0	+	+	tr	Strongly positive

#### CONCLUSIONS

Pure glycerol is an ideal preservative for human serum intended for the Wassermann reaction. In order to prevent the serum from becoming anticomplementary, it must be heated to about 56 C. for 30 minutes and then be mixed with an equal volume of glycerol. Serum kept for more than a year gave practically the same results as when fresh and without preservative.

## ROCKY MOUNTAIN SPOTTED FEVER IN CALIFORNIA\*

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In 1906, Ricketts demonstrated the susceptibility of guinea-pigs to Rocky Mountain spotted fever by the injection of infected human blood. The fever, the duration of the disease, and the cutaneous phenomena, resembled closely these conditions as observed in man. During the same year, this investigator proved that the wood-tick (*Dermacentor venustus*) was the intermediate host of spotted fever.<sup>1</sup> McClintic<sup>2</sup> and Fricks<sup>3</sup> later made extensive investigations of this disease in the Rocky Mountain regions of the United States.

As a result of the work of Ricketts in the establishment of this disease as an entity, both by inoculation into guinea-pigs and by transmission experiments through ticks, it became possible to determine the area of prevalence of spotted fever throughout the western states, where it is now recognized that this seasonal disease has an irregular incidence. The occurrence of the disease in California has been recognized, as is shown by Kelly,<sup>4</sup> who collected the histories of 32 cases as having occurred in the last 14 years. It was not, however, until June, 1916, that the occurrence of this disease in California was established definitely by animal experimentation.

### OXNARD CASE

On May 31, 1916, this bureau was called on to investigate a case of suspected typhus fever at Oxnard, Ventura County. The patient had been sick about 12 days. There had been some bronchial involvement, fever at times at 106, and pronounced coma. There was a maculopetechial eruption, which involved practically the entire body covering. The most extensive involvement was on the abdomen, thorax, and thighs. Figure 1, 15th day of illness shows the extent of the skin eruption. The recovery was gradual, extending over about 8 weeks, and complicated by extensive bedsores. No body lice were found on the patient or his clothing; furthermore, after his recovery it was learned that he had been bitten by a tick about 2 weeks before his illness.

\* Received for publication July 9, 1917.

<sup>1</sup> Jour. Am. Med. Assn., 1906, 47, p. 358.

<sup>2</sup> Pub. Health Rep., 1912, 27, p. 732.

<sup>3</sup> Ibid., 1913, 28, p. 1647; 1915, 30, p. 15.

<sup>4</sup> California State Jour. Med., 1916, 14, p. 407.

Only a single tick could be obtained from the vicinity of the patient's home, and this proved to be the variety (*Dermacentor venustus*) responsible for the transmission of Rocky Mountain spotted fever.

*Inoculations.*—Eight c.c. of blood drawn from the patient were injected intraperitoneally in a rhesus monkey. Chart 1 shows a temperature curve of 10 days' duration in this animal. The fever remained high, there being noticeable remission only on the 2 days when the animal was anesthetized and bled. The termination was by crisis extending over about 36 hours. The slight fever following the crisis is accounted for by complications—necrosis and sloughing.



Fig. 1. Oxnard Case, showing maculopetechial eruption.

On the 7th day of illness, subcuticular hyperemic areas appeared on the face and ears; at this time these could be easily pressed out, and were only mildly congested areas of varying size. On the 10th day of illness, however, these lesions, as also lesions which appeared on the perineum and scrotum, had become petechial in character. Figure 2, taken after the crisis, shows the extent of the maculopetechial lesions of the face and ear.

The skin lesions of the left ear, scrotum, and left hind knee terminated in extensive necrosis and sloughing, and were slow in healing.

The necrosis of the scrotum and left hind knee was of considerable extent, the former covering two thirds of the scrotal area and involving the deeper subcutaneous tissues. This latter was an irregular area, 2.5 cm. in diameter, and involved only the superficial structures.

On the 1st day of fever, the monkey was bled from the heart, and 15 c.c. of blood taken. The blood was defibrinated and 2 c.c. and 0.5 c.c. injected

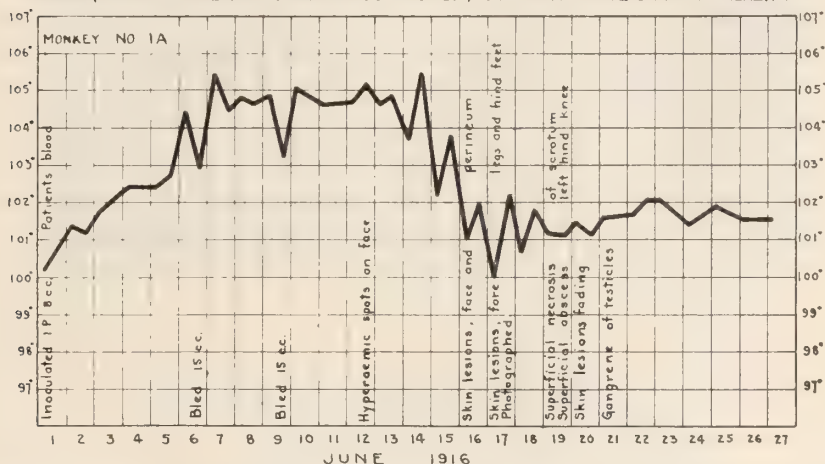


intraperitoneally into 2 male guinea-pigs, respectively. These animals both showed a temperature of over 104 on the 4th day. The curves ranged from 104 to 106 F. for 6 and 9 days, respectively, and terminated by crisis, followed by death. The scrotum of 1 was somewhat swollen on the 5th day of temperature. On each succeeding day, the inflammation became more marked, and at the time of death, on the 9th day, the edema was so extensive that the scrotum was 4 times its normal size. Aside from the general involvement of the scrotum, there were several macular spots which could be readily seen through the white skin, and which were readily obliterated by pressure. On postmortem, the only changes were a slight enlargement of the spleen, the axillary, and inguinal lymph nodes.

The 3rd generation of this strain of Rocky Mountain spotted fever in laboratory animals was established by transferring blood from the 2nd generation immediately on the appearance of fever. This—the Ventura County—

### ROCKY MOUNTAIN SPOTTED FEVER, OXNARD, VENTURA CO. STRAIN.

INVESTIGATIONS OF BUREAU OF COMMUNICABLE DISEASES, CALIFORNIA STATE BOARD OF HEALTH.



strain of virus has been passed through 80 generations in guinea-pigs. It is now being maintained in ticks.

The following conclusions may be drawn from a study of the records of these 80 passages:

The virulence of the virus could be increased or decreased depending on the relation of the time of transfer to the onset of fever. A temperature of 104 could be made to appear on the 2nd day, by transferring the blood of several successive generations on the 1st day of fever. By making the transfer late in the febrile stage, the incubation period could be prolonged 8-10 days. In 1 instance only was there a 17-day incubation period.

The more prolonged the incubation period, the more pronounced was the swelling of the scrotum or vulva. The swelling appeared about the 3rd day of fever and progressed rapidly. In white-skinned males, a generalized infiltration was noticeable in the scrotal covering, and this was often supplemented by macular spots, which also appeared on the feet. Postmortem revealed swell-

ing and punctate hemorrhages in the lymph nodes, enlarged and cyanosed spleen.

By injecting a virus of low virulence the scrotal involvement could be made to appear in 100% of the animals.

The size of the dosage (0.5 c.c.-4.0 c.c.) did not influence the incubation period, except in the case of a generation of virus of low virulence. Such a virus required a large dose to produce infection.



Fig. 2. Oxnard Strain of Virus in monkey, showing maculopetechial lesions of skin and necrotic area of nose.

#### PALO ALTO CASE

While the following case is included in the incidence of this disease in California, the history indicates that the infection took place in Nevada.

The patient had left his home in Palo Alto, April 30, 1917, for Rogers, Idaho. He remained there until May 3, at which time he traveled by team to Jarbidge, Nevada. While making this journey it was necessary for the patient and other members of the party to cut sage brush to fill in almost impassable roads. In visiting the mines in and around Jarbidge, he daily rode horseback. Although he contended he was exposed to tick bite only when cutting brush on the way to Jarbidge, it must be remembered that ticks infest horses and his daily riding afforded opportunity for infection.

On returning from Jarbidge to Rogers, May 11, the patient noticed a pimple on the skin over the sternum. This lesion probably represents the focus of infection, an infected tick having gained entrance through the opening of the shirt.

On May 12, at Rogers, the patient had chilly sensations, rapidly followed by flushed face and general sensation of fever, with intense pains in the muscles and bones, no appetite, and some nausea. On arriving at his home

in Palo Alto, May 14, the aching of the bones became less severe, but an intense headache developed. At this time the temperature was 103, the respiration 18, the pulse 82. From this time there was a constant severe headache. The eruption appeared first on the inner surfaces of the thighs, May 16, 1917, and extended within 2 days to the entire body. The surfaces of the hands



Fig. 3. Palo Alto Case; maculopetechial spots.

and feet were, however, not as extensively involved as the other surfaces. It will be noted that the order of appearance of the eruption in this case is the reverse to that of the usual case of spotted fever.

On the 3rd day of the eruptive stage there were both mulberry and maculopetechial spots. The new mulberry spots, representing hyperemic areas could be easily pressed out; while the mature maculopetechial spots, representing



Fig. 4. Palo Alto Case; maculopetechial spots.

endarteritis and interstitial extravasations could not be pressed out. The mulberry spots were about 0.4 cm. in diameter, with irregular borders, not elevated, while the petechial spots, somewhat larger, varied 0.4-1.2 cm. and were irregular in outline and apparently somewhat elevated. The spots remained discrete and gave the body a speckled appearance (Figs. 3 and 4).

Neither the spleen nor the liver was palpable. Prior to the eruptive stage there was considerable albumin in the urine. This decreased in amount and finally disappeared about the 12th day of illness. The leukocyte count was slightly above normal. The patient was at all times conscious, although speech was labored. The eyes were somewhat injected and sensitive to light. The fever was at no time above 103, and it began to fall by crisis just prior to the appearance of the eruption. It is significant that the respiratory rate rose with the fall in temperature—a syndrome common in Rocky Mountain spotted fever.

On the whole it may be said that this was a mild case of Rocky Mountain spotted fever; the fever was not high and there were moderate daily remissions. The most prominent subjective symptoms were intense pains in the bones during the first 3 days, followed by severe continuous headache for 10 days. The large number of spots were somewhat out of proportion to the symptoms.

*Inoculations.*—May 20, 1917, 10 c.c. of blood were drawn from the median vein of the arm, and inoculated intraperitoneally in 2 male guinea-pigs, 2 c.c. and 0.5 c.c., respectively. May 26, the animal which received the larger dose showed some swelling of the scrotum. The following day the scrotum had enlarged to twice its normal size; the swelling was accompanied by hyperemia, which was not only general, but intensified in numerous small areas. At no time did the temperature in this animal rise much above normal, and had the temperature curve alone been relied on as an indication of infection, the changes in the scrotum might easily have been overlooked.

As this change in male guinea-pigs is a means of differentiating Rocky Mountain spotted fever from typhus fever, and as these 2 diseases are the only ones with which we are concerned in the diagnosis of the case in question, it may be definitely concluded that this case was one of Rocky Mountain spotted fever.

#### CONCLUSIONS

The results of animal inoculations in these cases definitely establish the occurrence of Rocky Mountain spotted fever in California.

The finding of *Dermacentor venustus* in Ventura County and the occurrence of a case there marks that region as a new area of possible prevalence of the disease in California.

Advantage should be taken of the inoculation test to establish definitely the nature of the seasonal eruptive fever which occurs in Modoc and Lassen counties.

# COMPLEMENT FIXATION WITH A SPECIFIC ANTIGEN IN ACUTE POLIOMYELITIS \*

M. NEUSTAEDTER AND E. J. BANZHAF

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Several attempts have been made by various investigators to obtain a specific antigen for complement fixation in poliomyelitis, so far without success.

We have obtained a specific antigen by digesting poliomyelitic virus with trypsin. A 5% suspension of brain and cord of monkeys that have died of poliomyelitis is filtered through a Berkefeld or Heim filter, sterile water being used as the menstruum. The trypsin is added in proportion of 1:50 and permitted to act at room temperature for three hours; 0.4% tricresol is then added to stop further action by the trypsin.<sup>1</sup>

The poliomyelitis antigens were diluted 1-5, the spinal fluid was used in 10 times the amount of the serum. The tubes were incubated as a rule for 2 hours in the water bath at 37 C. Earlier in the work, the antigen was used in less than one-fourth and later less than one-half of the anti-complementary dose. It is not inclined to be anticomplementary and was tested with each reagent alone, and these controls gave no hemolysis.

The bacterial antigens used as controls in our study of complement fixation in poliomyelitis were prepared by the methods employed by Miss M. A. Wilson in this laboratory, who made all the tests.

A saline emulsion of the dried organisms is made and the standard dose determined by titration with a homologous serum of known titer. The standard dose of each antigen contains 2 fixation units and one-fourth, or less, of the anticomplementary dose. The meningococcus tests are held for 30 minutes in the water bath at 37 C.

The tuberculosis and streptococcus tests are held for 2 hours in the water bath at 37 C. The antigen for the Wassermann tests was a cholesterinized alcoholic extract of guinea-pig-heart or ox-heart, made by Dr. Hadsopulos of the Wassermann department of Bellevue Hospital. The tubes were kept 1 hour in the water bath at 37 C.

In all of the tests 2 units of complement and 2 units of amboceptor were used, the cells having been sensitized with the amboceptor before they were added; 0.1 c.c. of a 5% suspension of sheep's corpuscles was used for indicator of the reaction of fixation.

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<sup>1</sup> For more elaborate description of the preparation, see Neustaedter and Banzhaf, Jour. Am. Med. Assn., 1917, 68, p. 1531.



No tests were made without controls of the serum or cerebrospinal fluid for anticomplementary action and for natural antisheep amboceptor.

All antigens were tested as to the anticomplementary dose, as to fixation with a homologous serum, and as to non-specific fixation with a heterologous serum.

The complement was a pool of the serum of from 6 to 10 pigs in every set of tests. The serum from each pig had been tested before pooling for natural antisheep amboceptor, for specific fixation with each control antigen-and-serum-combination to be used in the tests, for hemolysis of 0.1 c.c. of a 5% suspension of sheep's erythrocytes with the Wassermann unit of antisheep amboceptor.

All readings were made when the controls of the hemolytic system and of anticomplementary reaction in serums, fluids and antigens were completely laked. The tubes with positive reactions were then replaced in the water bath for one hour, to verify the accuracy of the reading. The reports were made on the first reading, but in most instances the reactions remained unchanged until the end of the hour.

In control of our work, we have used spinal fluid and serum of patients with other diseases than poliomyelitis. We also tested the same fluids and serums with various antigens at the same time, in order to be certain that we have a specific antigen.

We have examined 152 spinal fluids and 60 blood serums. The ages of the poliomyelitis patients ranged between  $2\frac{1}{2}$  and 23 years. The cases were frank and suspected, in the febrile and afebrile stages. The duration of the disease was from 1 to 40 days.

Of the 55 spinal fluids of poliomyelitis patients (Table 1) we obtained 2 + + + +, 1 + + +, 9 + +, 11 +, 12  $\pm$ , 7 —, the balance were anticomplementary, due in some cases to their long standing after removal. The chemical and cytological results as well as the clinical symptoms in the doubtful, weakly and strongly positive cases coincided with the results as to fixation. Two of the negative cases proved on necropsy to be tuberculous meningitis; one negative case showed a + + + + Wassermann in the spinal fluid as well as in the blood.

Ed. S., a boy, aged 9, became ill with a temperature of 102 F. at 4 a. m. with malaise, and had a profuse diarrhea; the temperature rose to 104 F. within a few hours and toward evening he became stuporous and had convulsions. The spinal fluid was under high pressure at 9 p. m., contained 14 cells per c.mm., mostly lymphocytes, albumin + + +, globulin + + +, Fehling's reduction + +, no bacteria on smear or on culture; it was negative to cholesterin, tuberculous and meningococcus antigens. These results were obtained from examinations on the subsequent days. There was a slight left hemiparesis and a tendency to the Babinski phenomenon in the left foot. The pupillary reflex was normal, while the pupils were dilated. The deep and superficial reflexes were exaggerated. There was no Kernig or Macewen sign. No rigidity of the neck. The child could not be aroused from his stupor. He had a bloody stool. A tentative diagnosis of poliomyelitis was agreed on and 5 c.c. of immune

TABLE 1

SPINAL FLUIDS OF FRANK AND SUSPECTED CASES OF POLIOMYELITIS AND FIXATION  
WITH POLIOMYELITIS AND OTHER ANTIGENS

Number	Symptoms	Albu- min	Cells	Glob- ulin	Sugar	Poli- mye- litis Anti- gen	Was- ser- mann	Tuber- cle Anti- gen	Strep- to- coccus Anti- gen	Menin- go- coccus Anti- gen
1	Weakness in ex- tremities; hydro- cephalus	+++	Moderate increase	+++	+++	+++	0	0	0	0
2	Spastic hemiplegia	+++	Great increase	+++	+++	++	0	0	0	0
3	.....	+	Normal	+	+++	+	0	0	0	0
4	Meningism; ataxia	+	Slight increase	+	+++	+	0	0	0	—
5	Deltoid paralysis; convulsions	+	Slight increase	+	+++	+	—	0	0	—
6	Meningism	+	Slight increase	+	+++	±	0	0	0	—
7	Paraplegia; re- covered	+	Slight increase	++	+	—	0	0	0	0
8	Paresis	+	Moderate increase	+	+++	—	0	0	0	0
9	Deltoid paralysis	+	Slight increase	+	++	++	0	0	0	0
10	Measles; paresis	+	Slight increase	+	+++	+	0	0	0	0
11	Paraparesis	+	Slight increase	++	++	±	0	0	0	0
12	Meningism	+	Normal	+	+++	±	0	0	0	0
13	No data	++	0	++	++	+	0	0	0	0
14	No data	+	Slight increase	+	+++	±	—	—	±	±
15	Scarlet fever; tran- sient blindness; meningism	+	Normal	+	+++	++	—	±	—	—
16	Meningism	+	Slight increase	+	+++	++	±	±	±	—
17	Flaccid paralysis	++	Slight increase	++	+++	++	—	±	—	—
18	No data	+	Slight increase	+	+++	++	0	0	0	0
19	No data	+	Slight increase	±	+++	±	+	±	±	++++
20	Paralysis of neck muscles	+++	Slight increase	+++	+++	++	—	±	—	++++
	Same case	++	Slight increase	+++	+++	±	—	±	—	—
21	No data	+	Slight increase	+	+++	+	0	—	0	0
22	Meningism	+	Normal	+	+++	+	—	0	0	0
23	Bulbar paralysis	++	0	++	+++	+++	—	0	0	0
24	Measles; pertussis; meningism	+	Normal	±	+	+	—	—	0	0
25	Left facial par- alysis	+	Normal	±	+++	+	—	—	—	—
26	Paralysis of right arm	+	Normal	±	+++	+	—	0	0	0
27	Tuberculous men- ingitis	+++	Great increase	+++	+	++	±	±	±	—
28	Bulbar paralysis	+++	0	+++	+++	+++	—	—	0	0
29	Measles; foot drop	++	0	+	+++	—	0	0	0	0
30	Ataxia	+++	Slight increase	+++	+++	—	++++	0	0	0
31	No data	±	Slight increase	±	+++	±	—	—	0	0
32	Paraplegia	+++	Moderate increase	±	++	+	—	—	0	0
33	Paraplegia	.....	Moderate increase	.....	.....	±	—	—	0	0
34	Paraplegia	+++	Great increase	+++	+	±	—	—	0	0
35	Paraplegia	++	Moderate increase	++	++	±	—	—	0	0
36	Deltoid paralysis	+	Slight increase	+	+++	±	—	—	0	0
37	Weakness of right arm; ataxia	±	Slight increase	±	+++	±	—	—	0	—
38	Tuberculous men- ingitis	+++	Great increase	+++	Trace	—	0	0	0	—
39	Convulsions; stupor	+++	Moderate increase	+++	++	—	—	—	0	—
40	Deltoid paralysis	++	Moderate increase	++	+++	+	0	0	0	0
41	Tuberculous men- ingitis	+++	Great increase	+++	+++	—	0	0	0	0

horse serum were injected intraspinally. Within 1½ hours a severe reaction followed, but after an injection of morphin (¼ grain) the child became quiet. He seemed to be doing fairly well, when at 4:30 a. m. he died suddenly of respiratory failure.

The picture of this case does not coincide with poliomyelitis, and this is further confirmed by the negative reaction of the spinal fluid, since the 2 cases that gave a ++++ reaction died, and in every case with high fever we had ++. However, in view of the absence of a necropsy we have classed the case as one of poliomyelitis. The other negative cases were recovering, and it is possible that when cases recover, the fluids become negative.

Of 15 spinal fluids from cases of tuberculous meningitis (Table 2), 7 proved negative with the poliomyelitis antigen, 1 ±, and 1 +, 6 were anticomplementary.

Of 20 spinal fluids of cases of epidemic cerebrospinal meningitis (Table 3), 13 were negative, 3 doubtful and 4 anticomplementary.

Of 22 spinal fluids of cases of tertiary syphilis, one gave a ++++ reaction with the poliomyelitis antigen, 1 +, 3 were anticomplementary, and the rest negative (Table 4).

Of 40 spinal fluids of normal and diverse pathologic conditions, one gave a ++++ reaction with the poliomyelitis antigen, 7 were anticomplementary, and the rest negative (Table 5).

Of 60 blood serums of various pathologic conditions, none gave a positive reaction with the poliomyelitis antigen (Table 6).

The results of the tests show that of 42 spinal fluids of frank and suspected cases of poliomyelitis, 23, or 53.5%, gave a positive reaction, 12, or 27.9%, gave a doubtful one, and 4, or 9.5%, a negative reaction. When we take into consideration the fact that the doubtful reactions were associated with clinical symptoms and cytological and chemical reactions in the fluids, showing unmistakably that an inflammatory process was going on in the meninges, we must place some value on these results.

We have had only 2 blood specimens from patients with poliomyelitis, and both were negative to the poliomyelitis antigen. The spinal fluid of 1 of these cases (1052) was + and the other (866) negative. Of course, no conclusion can be drawn from this. It is significant, however, that with 1 exception, Case 8972, which gave a ++++ reaction with the meningococcus antigen, all serums gave negative reactions with bacterial antigens. Further studies may bring more light into this question.

TABLE 2

SPINAL FLUIDS OF CASES OF TUBERCULOUS MENINGITIS AND FIXATION WITH POLIO MYELITIS AND OTHER ANTIGENS

Number	Cells	Albu- min	Glob- ulin	Sugar	Polio- mye- litis Anti- gen	Was- ser- mann	Tuber- cle Anti- gen	Strep- to- coccus Anti- gen	Menin- go- coccus Anti- gen
1	Great increase	+++	+++	+++	—	0	0	0	0
2	Blood	++++	++++	.....	—	0	0	0	0
3	Great increase	+++	++++	Trace	—	0	0	0	—
4	Slight increase	++	++	+	—	0	0	0	0
5	Great increase	++++	++++	++++	±	—	±	±	—
6	Great increase	++	++	+	—	±	±	±	—
7	Moderate increase	++	++	+++	—	—	—	0	0
8	Great increase	+++	+++	+	++	±	±	±	—
9	Great increase	+++	+++	Trace	—	0	0	0	0

TABLE 3

SPINAL FLUIDS OF CASES OF EPIDEMIC MENINGITIS SHOWING REACTION TO POLIOMYELITIS ANTIGEN

Number	Cells	Albumin	Globulin	Sugar	Poliomyelitis Antigen
1	Blood	++++	++++	+++	—
2	Great increase	+++	+++	++	—
3	0	++	+	++	±
4	Great increase	+++	+++	+	—
5	Moderate increase	++++	++++	+++	±
6	Great increase	++++	++++	++	—
7	Great increase	++++	++++	+++	—
8	Great increase	+++	+++	+	—
9	Great increase	+++	+++	+	—
10	Great increase	+++	+++	+	—
11	Great increase	+++	+++	Trace	—
12	Great increase	++++	++++	+	—
13	Great increase	+++	+++	—	—
14	Great increase	+++	++++	+	—
15	Great increase	+++	+++	+	—
16	Great increase	+	±	+++	±

TABLE 4

SPINAL FLUIDS OF CASES OF TERTIARY SYPHILIS SHOWING REACTIONS WITH CHOLESTERIN AND POLIOMYELITIS ANTIGEN

Number	Diagnosis	Wassermann	Poliomyelitis Antigen
1	Tabes	++++	—
2	Cerebrospinal lues	++++	±
3	Combined sclerosis	++++	±
4	Tabes	++++	±
5	Tabes	++++	±
6	Tabes	++++	±
7	Tertiary lues	++++	++++
8	Cerebrospinal lues	++++	+
9	Cerebrospinal lues	++++	—
10	Cerebrospinal lues	++++	—
11	Cerebrospinal lues	++++	±
12	Cerebrospinal lues	++++	—
13	Cerebrospinal lues	—	—
14	Tabes	++++	—
15	Tabes	—	—
16	Tertiary lues	—	—
17	Cerebrospinal lues	++++	—
18	Tabes	++++	—

TABLE 5

SPINAL FLUIDS OF MISCELLANEOUS CASES AND FIXATION WITH POLIOMYELITIS AND OTHER ANTIGENS

Number	Diagnosis	Cells	Albumin	Globulin	Sugar	Poliomyelitis Antigen	Wassermann	Tubercle Antigen	Streptococcus Antigen	Meningococcus Antigen
1	Normal	.....	.....	.....	.....	—	—	—	—	—
2	Meningism	Normal	.....	.....	+++	—	—	—	—	—
3	Childbirth	.....	.....	.....	.....	—	—	—	—	—
4	Cerebral hemorrhage	.....	.....	.....	.....	—	—	—	—	—
5	Meningitis	.....	.....	.....	.....	—	—	—	—	—
6	Cerebral hemorrhage	.....	.....	.....	.....	—	—	—	—	—
7	None	.....	.....	.....	.....	—	—	—	—	—
8	Normal	—	—	—	+	—	—	—	—	—
9	Tetany	.....	.....	.....	.....	—	—	—	—	—
10	Normal	—	—	+	+	—	—	—	—	—
11	Normal	—	—	—	+	—	—	—	—	—
12	Normal	—	—	—	+	—	—	—	—	—
13	Staphylococcus meningitis	Great increase	++++	++++	—	—	—	—	—	—
14	Brain abscess	Great increase	++++	++++	Trace	—	—	—	—	—
15	Brain hemorrhage	Great increase	+++	+++	+	—	—	—	—	—
16	Tumor hypophysis	.....	.....	.....	.....	—	—	—	—	—
17	Epilepsy	.....	.....	.....	.....	—	—	—	—	—
18	Normal	.....	.....	.....	.....	—	—	—	—	—
19	?	Great increase	+	+	+++	—	—	.....	—	—
20	?	Normal	++	++	+++	—	.....	.....	—	—
21	Meningism	.....	.....	.....	.....	—	—	—	—	—
22	Normal	Normal	±	±	+++	—	—	—	—	—
23	Meningism	Slight increase	+	+	+++	—	—	—	—	—
24	Pertussis	Normal	+	±	+++	—	—	—	—	—
25	?	Normal	+	±	+++	—	—	—	—	—
26	Staphylococcus meningitis	Great increase	+++	+++	+++	—	—	—	—	—
27	Meningism	Slight increase	+	+	+++	—	—	—	—	—
28	Brain hemorrhage	Blood	++	++	+++	—	—	—	—	—
29	?	Great increase	+++	+++	+	—	—	—	—	—
30	Meningism	Slight increase	++++	++++	+++	—	—	—	—	—
31	Meningism	Normal	±	±	+++	—	—	—	—	—
32	Interstitial nephritis	Normal	.....	+	.....	++++	—	—	—	—
33	Pneumonia; meningism	.....	.....	.....	.....	—	—	—	—	—

As controls, we have tested spinal fluids of normal and various pathologic conditions with the poliomyelitis antigen. Of 97 such fluids, 2 prove + + + +, 1 + +, 1 +, 9 ±, and the balance negative. Cases 6 and 90, which were positive to the poliomyelitis antigen, gave a strongly positive Wassermann reaction. We explain this discrepancy by the fact that at that time we filtered our suspension through paper pulp and much of the lipid substance remained in the filtrate. The reactions were in all probability due to the presence of the lipid substance.



TABLE 6  
SHOWING FIXATION OF BLOOD-SERUM WITH VARIOUS ANTIGENS

Diagnosis	Polio- myelitis Antigen	Wasser- mann	Tubercle Antigen	Strepto- coccus Antigen	Meningo- coccus Antigen
Chorea.....	—	—	—	0	—
Tabes.....	—	++++	—	0	—
Lues.....	—	++	0	0	—
Poliomyelitis.....	—	0	0	0	—
Epidemic meningitis.....	—	0	0	0	—
Poliomyelitis.....	—	0	0	0	—
General paresis.....	—	—	0	0	0
Nephritis.....	—	—	0	0	0
Mania.....	—	++++	—	—	—
Constitutional inferiority.....	—	±	—	—	—
Mania.....	—	—	—	—	—
Cerebrospinal lues.....	—	++++	—	—	—
Cerebrospinal lues.....	—	++++	—	—	—
Arteriosclerosis.....	—	±	—	—	—
Cerebrospinal lues.....	—	++++	—	—	—
Tertiary lues.....	—	++++	—	—	—
Secondary lues.....	—	++++	—	—	—
Gout.....	—	—	0	0	0
Pleuritis.....	—	—	0	0	0
Suppurative salpingitis.....	—	—	0	0	0
General paresis.....	—	—	0	0	0
Tertiary lues.....	—	++++	0	0	0
Cerebrospinal lues.....	—	++++	0	0	0
Cardiovalvular disease.....	—	—	0	0	0
Leukemia.....	—	—	0	0	0
Nephritis.....	—	—	0	0	0
Normal.....	—	—	0	0	0
Polyneuritis.....	—	—	0	0	0
Cardiovalvular disease.....	—	++++	0	0	0
Cardiac disease.....	—	—	0	0	0
Normal.....	—	—	0	0	0
Hemiplegia.....	—	—	0	0	0
Suppurative salpingitis.....	—	—	0	0	0
Cerebrospinal lues.....	—	+	—	—	—
Poliomyelitis.....	—	++++	+	—	—
Apoplexy.....	—	—	—	—	—
Nephritis.....	—	—	—	—	—
Normal.....	—	—	—	—	—
Cholelithiasis.....	—	—	—	—	—
Abortion.....	—	—	—	—	—
Pneumonia.....	—	—	—	—	—
Lues.....	—	±	—	—	—
Gonococcal arthritis.....	—	±	—	—	—
Cholelithiasis.....	—	—	—	—	—
Normal.....	—	—	—	—	—
Normal.....	—	—	—	—	—
Lues.....	—	++++	—	—	—
Nephritis.....	—	—	—	—	—
Brain tumor.....	—	—	—	0	0
Lues.....	—	+	—	0	0
Cardiovalvular disease.....	—	++++	—	—	—
No diagnosis.....	—	—	—	—	—
Tabes.....	—	++++	—	—	—
Hydrocephalus.....	—	—	—	—	—
Lues.....	—	++++	—	—	—
Salpingitis.....	—	—	—	—	—
Aortitis.....	—	++++	—	—	—
Epididymitis.....	—	—	—	—	++++
Alcoholism.....	—	—	0	0	0

We have since filtered all suspensions through Berkefeld or Heim filters and the results as recorded show the correct interpretation. All serums of syphilitic patients with a strongly positive Wassermann reaction were negative to the poliomyelitis antigen. Case 8991, which shows

a ++++ reaction with our antigen, was diagnosed as an interstitial nephritis. The patient was 37 years old and had been only a few days in the hospital before he died. He had pain in the legs; the temperature ranged between subnormal and 101 F.; patellar reflexes sluggish, and achilles reflexes absent. At necropsy there was found interstitial nephritis, but the cerebral nervous system was not examined. The cells in the fluid were reported as 4 per c.mm. and the globulin as +; no Fehling's test or tests for albumin were made. In view of these incomplete findings no conclusions can be drawn.

#### CONCLUSION

It would appear that an antigen has been found, which probably is specific and fixes complement, hence of positive diagnostic value in poliomyelitis. This result is in accord with the production of immune bodies in a horse injected with poliomyelitic virus digested with trypsin as described.

# COMPARATIVE VALUE OF METHODS OF PREPARING POLLEN ANTIGEN

RALPH OAKLEY CLOCK

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The methods used to prepare pollen extract have not been at all uniform. Noon<sup>1</sup> extracted the pollen with distilled water, aided by repeated freezing and thawing, according to Dunbar's original method. Freeman<sup>2</sup> adopted the same procedure. Clowes<sup>3</sup> precipitated the pollen with acetone and extracted in water. Lowdermilk<sup>4</sup> extracted the pollen in physiologic salt solution with 0.5% phenol. Koessler<sup>5</sup> employed 8.5% salt solution for the extract which he sometimes precipitated with 10 times its volume of 95% alcohol. Goodale<sup>6</sup> prepared extracts by soaking the pollen in 15% alcohol.

In view of the diversity of methods used, it seemed advisable to investigate the relative merits of each, in order to determine the most efficient method of extraction. Accordingly, on July 16, 1915, we prepared a series of pollen antigens by various methods.

## PREPARATION OF ANTIGENS

A mixture was made of equal parts by weight of the dried pollens of timothy, red top, June grass, orchard grass, rye, sorrel dock, daisy, maize, ragweed, and goldenrod. A definite quantity of this mixture was extracted in different solvents, using the technic described in a previous article.<sup>7</sup> In each case, the proportions used gave a dilution such that 1 c.c. was equivalent to approximately 14,000 units of pollen. The pollen unit was established by Noon as the equivalent of one millionth gram of pollen.

*A.*—1.0156 gm. of the pollen mixture were extracted in 72.6 c.c. of a diluent, consisting of 66⅔% glycerol and 33⅓% saturated sodium chlorid solution.

*B.*—To 10 c.c. of Antigen A was added 0.054 c.c. of 92% phenol, making it 0.5% phenol.

*C.*—1.008 gm. of the pollen mixture were extracted in 72 c.c. of 66⅔% glycerol.

*D.*—To 10 c.c. of Antigen C was added 0.054 c.c. of 92% phenol, making it 0.5% phenol.

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<sup>1</sup> Lancet, 1911, 1, p. 1572.

<sup>2</sup> Ibid., 1911, 2, p. 814.

<sup>3</sup> Pro. Soc. Exper. Biol. and Med., 1913, 10, p. 70.

<sup>4</sup> Jour. Am. Med. Assn., 1914, 63, p. 141.

<sup>5</sup> Forchheimer's Therapeutics of Internal Diseases, 1914, 5, p. 671.

<sup>6</sup> Boston Med. and Surg. Jour., 1915, 173, p. 42.

<sup>7</sup> Jour. Infect. Dis., 1917, 21, p. 387.

*E.*—0.5203 gm. of the pollen mixture was extracted in 37.2 c.c. of 9% sodium chlorid solution.

*F.*—0.5434 gm. of the pollen mixture was extracted in 38.8 c.c. of distilled water.

*G.*—1.0064 gm. of pollen mixture were extracted in 71.9 c.c. of 15% alcohol.

#### COMPLEMENT-FIXATION TESTS

The 7 antigens were each subjected to the complement-fixation test on July 19, 1915, in order to determine the relative antigenic power of the extracts. The antigens were titrated against known positive antipollen serums, obtained by immunizing rabbits with an increasing number of units of pollen. The same technic was employed as that adopted by the laboratories of the Department of Health of New York City for all complement-fixation titrations. The technic is indicated in Table 1, which shows the results of titrating Antigen A.

Table 1 shows that 0.000020 gm. of pollen is the antigen unit (smallest amount that gives complete fixation). Since the pollen unit is 0.000001 gm. of pollen, it follows from this table that 1 pollen unit is equivalent to  $\frac{1}{20}$  of a unit of Antigen A. This table also shows that the antigen caused complete fixation of complement in a dilution of 1:500 (0.20 c.c. of 1:100).

TABLE 1  
TITRATION OF ANTIGEN A (GLYCEROL-SALT EXTRACT)\*†

Tube	Immune Serum, C.c.	Antigen		10 % Complement, C.c.	0.9 % Salt, C.c.	Sensitized Erythrocyte Suspension, C.c.	Results
		1:100 C.c.	Grams of Pollen				
1	0.01	0.25	.000025	0.1	0.0	0.2	++++
2	0.01	0.20	.000020	0.1	0.0	0.2	++++
3	0.01	0.15	.000015	0.1	0.05	0.2	+++
4	0.01	0.10	.000010	0.1	0.10	0.2	++
5	0.01	0.05	.000005	0.1	0.15	0.2	—
6	0.01	0.025	.0000025	0.1	0.20	0.2	—
7	0.0	0.4		0.1	0.0	0.2	—
8	0.0	0.3		0.1	0.0	0.2	—
9	0.0	0.2		0.1	0.05	0.2	—
10	0.0	0.1		0.1	0.10	0.2	—
11	0.0	0.05		0.1	0.15	0.2	—
12	0.02	0.0		0.1	0.20	0.2	—

\* Mixtures of immune serum, antigen, and complement were placed in icebox for 15 hrs. before adding the suspension of sensitized erythrocytes. Results read after 1 hr. at 37 C.

† Citron's standard for the strength of a reaction is used in this table; namely, complete absence of hemolysis is indicated by a 4-plus sign (++++); faint hemolysis is shown by a 3-plus sign (+++); partial hemolysis is represented by a 2-plus sign (++); while a minus sign (—) indicates complete hemolysis.

#### RESULTS

Taking a unit of antigen as the smallest amount that gives complete fixation in the hemolytic series, the ratio of each antigen unit to a unit of pollen was found to be as follows:

One pollen unit was equivalent to:

- 1/20 of a unit of Antigen A
- 1/20 of a unit of Antigen B
- 1/50 of a unit of Antigen C
- 1/50 of a unit of Antigen D
- 1/25 of a unit of Antigen E
- 1/150 of a unit of Antigen F,

but the unit of Antigen G was so small that it could not be measured even in the lowest dilution (1:40) used in the titrations, inasmuch as it did not fix completely, but caused only partial fixation of complement.

Expressed in the highest dilutions of the antigens that gave complete fixation of complement, the results were:

Antigen A fixed completely in a dilution of 1:500

Antigen B fixed completely in a dilution of 1:500

Antigen C fixed completely in a dilution of 1:200

Antigen D fixed completely in a dilution of 1:200

Antigen E fixed completely in a dilution of 1:400

Antigen F fixed completely in a dilution of 1:65

Antigen G did not fix completely even in a dilution of 1:40.

#### SUMMARY

All antigens were prepared with the same proportion of pollen and solvent, so that 1 c.c. of each extract was equivalent to 14,000 units of pollen.

The most complete extraction of the pollen was obtained by using a solvent composed of 66 $\frac{2}{3}$ % glycerol and 33 $\frac{1}{3}$ % saturated sodium chlorid. Such an extract in a dilution of 1:500 gave complete fixation of complement.

Pollen extracted in glycerol and salt produced antigens that were 25% stronger in antigenic properties than antigens prepared by extracting the pollen in salt solution only. The highest dilution of salt extracts that gave complete fixation of complement was 1:400.

Phenol played no part whatever in the extraction of the antigenic principle of the pollen; for the antigens fixed to the same degree, whether or not phenol was used as part of the solvent.

Glycerol alone did not extract all of the antigenic principle from the pollen; the glycerolated extract being only two fifths as strong as the extract prepared in glycerol and salt.

Distilled water gave an extract that was only about one eighth as strong as the glycerol and salt extract.



# CHRONIC ARTHRITIS IN SWINE

PLATES 10 AND 11

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Arthritis is a comparatively frequent lesion in domestic animals and occurs in association with other pathologic conditions which correspond quite closely with those in man. Thus, arthritis is met with in acute general infections, such as postpartum infections in cattle, and following umbilical infections in calves and foals, and in epidemic streptococcal infections in horses and cattle; in chronic local infections of the mammary gland in cattle and swine, chronic forms of arthritis are found; in other animals including swine, cattle and other domestic animals, chronic arthritis of one or more joints is seen without other clinically evident infections.

Chronic arthritis with varying degrees of enlargement and deformity of joints is met with in otherwise apparently healthy market hogs. Such chronic lesions usually involve one or two joints, and occur sporadically, or there may be several instances in one herd. The lesions may correspond quite closely with certain forms of chronic deforming arthritis in man, in which symptoms of general infection, and for considerable periods also, marked nutritional disturbances may be absent.

The present study includes an examination of joints from 21 cases of arthritis in market hogs, the degree of joint involvement of which varied from a slight enlargement of the joint to extensive disorganization and deformity, in some instances suggesting in external appearance chronic tuberculosis. We have ascertained the pathogenic properties of such organisms as were isolated, by experiments in animals of the same species, thereby avoiding the factor of abrupt change of the host of the invading organism. The importance of the species of the experimental host, in modifying lesions produced by bacteria, seems not always to have received the attention it deserves.

Of the organisms which have been found in infections with arthritis in domestic animals *Bacillus pyogenes* has received most attention. Glage<sup>1</sup> has collected the literature concerning acute and chronic

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<sup>1</sup> Kolle u. Wassermann, *Handbuch d. path. Mikroorganismen*, 1913, 6, p. 148.

infections of domestic animals. Ward<sup>2</sup> has well summarized the lesions produced by *B. pyogenes* in swine and cattle, and noted a case of arthritis with periarticular abscesses caused by this organism.

The opportunity to examine hogs suffering from chronic arthritis, to secure the joints for study, and to obtain young swine for experimental study of arthritis, we owe to Mr. A. G. Leonard of the Union Stock Yards, Chicago. We thank also Dr. S. E. Bennett, veterinarian of the Union Stock Yards, and the veterinarians of the U. S. Department of Agriculture stationed at the stock yards, and officials of Swift & Company for cooperation in obtaining material.

The joints for examination were obtained fresh from hogs on the killing floor of Swift & Company and transported to the laboratory for examination. This material presented several advantages for study. The joints came as a rule from otherwise healthy hogs, of which complete veterinary inspection was made for visceral lesions, and whatever pathologic changes were found noted. The joints were thus examined at a time in the course of the arthritis when they were still serviceable, and complete dissections, sections and cultures from fresh material were possible. It is evident, of course, that this material is a selected one in respect to the subject of arthritis in swine, since it came from a group of animals in which whatever disease was present had not materially interfered with the growth, fattening, and suitability of the hog for market.

#### REPORT OF CASES

1.—The right hock joint was spindle-shaped and swollen; the overlying skin was not changed. The capsular tissue was found everywhere much thickened, tough, fibrous, in places calcified, the surface cartilage of the head and socket was denuded in corresponding places and covered with granulation tissue. The synovial membrane was replaced by villous granulation tissue, markedly in the upper recesses. The posterior ligament was hard and calcified. The cavity of the joint contained a small amount of odorless, slightly turbid exudate. The tonsils of both sides were enlarged and showed several whitish-yellow spots 1-2 mm. in diameter. Microscopically the granulation tissue in the synovial villi showed plasma and round cells and new capillaries. In the part where the cartilage was denuded, the granulation tissue encroached on the bone marrow and contained numerous giant cells; proliferation of cartilage cells and new formation of bone were noticed at the edges. Gram-positive diplococci were found in the granulation tissue and bone marrow.

The sections of the tonsils showed several small cavities with granular contents, staining homogeneously violet with hematoxylin, surrounding which was dense connective tissue without any giant cells or epithelioid cells. Gram-positive diplococci and plump bacilli were present in the abscesses. The smears of the joint exudate and the granulation tissue contained gram-positive diplococci and fewer gram-positive and gram-negative, short bacilli. Cultures of dilutions of the exudate gave *Staphylococcus albus*, a hemolytic streptococcus, and gram-positive short bacilli. Inoculations from the tonsils on blood agar gave principally hemolytic streptococci.

2.—The right carpal joint was swollen; no sinus; a small amount of clear exudate; the cartilage was almost intact, except for a round area of ulceration of the socket of os naviculare; reddish, hyperemic granulation tissue beneath the capsule and in interosseal spaces; posterior ligament calcified.

<sup>2</sup> Cornell Veterinarian, 1917, 7, p. 29.

In the tough, scarlike connective tissue of the joint capsule were tags of granulation tissue with mucoid degeneration in some places; plasma cell infiltration marked. The edge of the denuded cartilage was rather sharply defined with no sign of bone regeneration. Gram-positive cocci in section of granulation tissue. The tonsils were small, no abscesses.

The smears from the joint exudate showed gram-positive diplococci and short bacilli. In fluid cultures, streptococci of short chain (8-12) and gram-positive short bacilli developed. On blood agar cultures from the exudate and subcultures from fluid mediums developed colonies of hemolytic streptococci but no bacilli. Carbol-fuchsin stains of smears and sections for tubercle bacilli gave negative results.

3.—Hock joint enormously swollen; two sinuses anteriorly, one with undermined edges. The sinuses led into a cavity filled with greenish-yellow pus, in places very thick. The marrow of the os cuneiforme was exposed inside this cavity, and the surface cartilage of the os cuboideum was replaced by edematous fibrous tissue. In the periarticular fibrous tissue were two areas of granulation tissue, with caseous spots in the center. The entire appearance resembled joint tuberculosis. The tonsils were enlarged with several rice-sized yellowish spots on the surface.

Microscopically the granulation tissue from bone and capsule showed new capillaries and infiltration chiefly with neutrophil polymorphous cells, in places round and plasma cells. Neither epithelioid nor giant cells were found. In sections of bone the granulation tissue was seen to have destroyed the cartilage and to have entered the marrow spaces; small islands of cartilage were surrounded by the granulation tissue; in the epiphyseal part the fatty marrow was replaced by cell infiltration. Gram-positive diplococci were found in numbers in the granulation tissue, especially in the edges toward the cavity.

The tonsils contained irregularly shaped small abscesses surrounded by hyaline fibrous tissue; neither giant cells nor epithelioid cells were found; the abscess contents showed relatively small number of gram-positive cocci; no tubercle bacilli.

Smears of the joint exudate contained a number of gram-positive diplococci; no tubercle bacilli (antiformin). Cultures gave minute hemolytic colonies of streptococci exclusively. Cultures of the tonsils gave hemolytic streptococci, large whitish colonies of gram-negative bacilli (colon) and one small reddish colony of yeast.

4.—Hock joint swollen and ankylosed; capsule thick; small amount of clear viscid fluid in joint cavity; hyperemic granulation tissue beneath the capsule in front; cartilage of the cuboid bone denuded in one small spot; other bones normal; tonsils normal.

Microscopically the villous proliferation in the synovia contained many capillary vessels and plasma cells; also a few gram-staining bacilli. Cultures sterile.

5.—Carpal joint swollen; beneath capsule a layer of granulation tissue, internal ligament torn away in opening joint taking with it bone which seemed to be undermined by granulation tissue. Articular surface fairly smooth, except in depths of depressions.

Gram-positive, short bacilli were scattered about in the granulation tissue; smears of joint fluid showed a few coccal and bacillary gram-positive organisms. On blood agar, minute, semitranslucent nonhemolytic colonies of gram-positive, short bacilli were obtained.

6.—Some enlargement of hock joint, due to capsular thickening; no disorganization; clear exudate; small amount of granulation tissue inside joint,

and microscopically proliferation of synovial tags which in some places were replaced by granulation tissue with much plasma cell infiltration; the superficial layer of cartilage involved in fibrillation and deeper layers showed cartilage cell proliferation; many giant cells in marrow spaces. Gram-positive, short bacilli in granulation tissue, and smears of exudate also showed a few such bacilli. The cultures gave minute, nonhemolytic colonies of gram-positive bacilli.

7.—Moderate sized elbow joint with some periarticular thickening, otherwise no deformity; but little change in the cartilage; small amount of granulations around internal ligament. In sections a few gram-positive short bacilli in granulation tissue; smears and cultures, however, gave no organisms.

8.—Hock joint, about the same as in Hog 7; smears and cultures showed no bacteria.

9.—Large stifle joint; some thickening of capsule and erosion of cartilage at more prominent points in joint, so that the bone was exposed; posterior part showed more granulation tissue than in front; microscopically the remnants of surface cartilage were surrounded by granulating fibrous tissue and at the edges of the ulcers was new formation of bony tissue; plasma cells in granulation tissue and many giant cells in bone marrow; gram-positive, short bacilli with some coccal forms in the tissue and smears, and on blood agar cultures nonhemolytic, minute, semitranslucent colonies of gram-positive bacilli.

10.—Six months old hog; right hock joint enormously swollen; two sinuses in skin over front of joint leading to bursae containing thick putty-like, greenish-gray or yellowish-gray pus; larger portion of enlargement due to areas of capsular thickening, in center of which are collections of thick pus; some of the bursae intact, not opening internally or into joint, except one which opened into joint; cartilage intact, no eburnation; areas of more recent inflammation with synovial exudate. Microscopically, the surface cartilage of several bones was denuded and replaced by granulating fibrous tissue with round cells and plasma cells; many giant cells in marrow, not of Langhans' type; no caseous degeneration; regional lymph glands show inflammatory reaction. Stains for tubercle bacilli negative. By Gram method bacteria of coccal form were demonstrated in the granulation tissue.

Guinea-pigs injected intraperitoneally with joint exudate after treatment with antiformin did not develop tuberculosis.

Smears of joint fluid and of lymph-nodes revealed the presence of gram-positive cocci and bacilli. On blood agar minute, slightly hemolytic colonies of gram-positive, short bacilli and stronger hemolytic colonies of gram-positive cocci were obtained. The latter developed short chains in fluid mediums.

11.—Carpal joint swollen, especially anteriorly; medium amount of cloudy serous exudate in cavity; granulation tissue filled up the interosseal spaces; surface of cartilage denuded at several points; in joint cavity two small pea-sized necrotic masses.

Nasal polypus on both sides but no sign of inflammation or exulceration; accessory sinuses normal.

Microscopically the necrotic masses were found to be pieces of degenerated bone tissue.

Gram-positive short bacilli were stained in sections and smears. Blood agar cultures gave nonhemolytic, minute colonies of gram-positive short bacilli.

12.—Slight swelling of carpal joint; capsular tissue slightly thickened, fibrous, moderate amount of slightly cloudy, yellowish, viscid fluid in joint cavity; synovial tags proliferated in anterior part, and markedly hyperemic. Cartilage



intact. The changes were those of a mild proliferative type of synovitis. There was some bloody muco-pus in the left upper nasal meatus and left maxillary sinus. Ethmoid and frontal sinuses larger than usual, but otherwise normal. Microscopically was found proliferation of synovial tags and granulation tissue in places beneath capsule. Sections and smears did not show any organisms. On blood agar cultures from the joint, however, minute nonhemolytic colonies of gram-positive bacilli developed.

13.—Right hock joint enormously enlarged with one large sinus in front. A large abscess in lateral periarticular tissue containing greenish-yellow pus, connected with joint cavity by two fistulae; capsular tissue thick, fibrous, in places edematous; the joint cavity filled with thick pus and granulating fibrous tissue; surface cartilage of several bones denuded; bone marrow replaced by granulation tissue; in some parts by caseous material. Microscopically there was found much destruction of cartilage and bone tissue; fatty marrow almost replaced by granulation tissue with numerous giant cells; many gram-positive diplococci were found in granulation tissue and smears of joint exudate; on blood agar small colonies of hemolytic streptococci in short chains developed.

14.—Hock joint swollen; capsule fairly thick; no marked exudate in cavity; granulation tissue lined the capsule; surface cartilage of some bones tough and fibrillated; gram-positive bacilli in tissue, smears and cultures.

15.—Stifle joint swollen; hock joint normal; capsule fairly thick; in cavity some slightly cloudy serous exudate; cartilage and bone not destroyed; no organisms in smears or cultures.

16.—Hock joint swollen; granulation tissue beneath the capsule, especially in front, filling up interosseal spaces; small amount of thick pus in cavity; cartilage denuded over opposing articulating surfaces; a small abscess with inspissated pus in bone. Microscopically there was found besides the destruction of cartilage and bone-tissues, new formation of bone with layers of osteoblasts. Smears and cultures gave gram-positive bacilli.

17.—Carpal joint swollen; capsule thick and fibrous; some clear viscid exudate; proliferation of synovial tags, more in front; no destruction of cartilage or bone. Sections, smears and cultures failed to reveal any bacteria.

18.—Hock joints of both sides swollen, ankylosed; conditions similar on both sides; bony union of tarsal bones with obliteration of interosseal spaces; between tibia and tarsum small amount of viscid fluid; granulation tissue within the capsule.

Accessory nasal sinuses free from inflammation; tonsils normal. Sections of granulation tissue and smears contained gram-positive bacilli. On blood agar minute nonhemolytic colonies of the same bacilli developed.

19.—Conditions about the same as 18. In sections, smears and cultures gram-positive short bacilli were obtained. On blood agar faintly hemolytic colonies of bacilli appeared.

20.—Carpal joints of both sides showed reddish vascular granulations, more marked about the periphery; cartilage intact; smears and sections contained short bacilli, gram-positive; blood agar plates gave minute nonhemolytic colonies.

21.—Hock joints of both sides swollen. On opening right joint thick greenish-white necrotic material was found outside it but communicating with cavity; much granulation tissue about joint; at one point the bone was softened and fragments of bone lay in the necrotic material. This joint suggested in appearance tuberculosis.



Similar changes in the left joint; the bone was but little involved, large peri-articular abscess along tendon sheath. Marrow spaces filled with granulation and fibrous tissue. Smears and sections of granulation tissue revealed gram-positive short bacilli and diplococci. On blood agar appeared hemolytic colonies of streptococci and nonhemolytic colonies of short bacilli. No evidence of tuberculosis. The tonsils were 1.5 x 3 cm. and contained many small yellowish, calcified nodules,  $\frac{1}{2}$  x 2 mm. in diameter. On culture of tonsils colonies of a hemolytic streptococcus developed.

Carbolfuchsin stains of sections and smears, serum cultures after antiformin treatment and intraperitoneal injection of guinea-pigs all failed to demonstrate tubercle bacilli.

#### SUMMARY OF OBSERVATIONS ON THE SPONTANEOUS DISEASE

Of 21 cases examined hemolytic streptococci were found in 2, bacilli in 9, to be discussed later, streptococci and bacilli in 4; 4 cases yielded no organisms in smears, sections and cultures, and in 2 bacteria were discovered in either smears or sections, but failed to grow in cultures.

Most of the 6 cases, which did not yield any organisms at all or not in cultures, showed comparatively slight pathologic changes; i. e., the joint exudate was clear or only slightly clouded and not much increased in quantity, the capsular thickening moderate, cartilage and bone not destroyed. Proliferation of synovial tags or granulation tissue beneath the capsule was the principal change. On the other hand, those cases from which streptococci or streptococci and bacilli together were obtained showed more marked changes in the joints than those with bacillary infection only. In all cases of the former there was enormous capsular thickening and cartilaginous destruction stood in the foreground. The cases resembling tuberculosis externally were mostly of such mixed infection. The joints in which organisms were found in sections or smears but not in cultures, were bacillary infections. The cases in which bacilli exclusively were found showed different stages, from mild to severe. In the mild cases the changes in the cartilage and bone were not marked; at most there was denudation of the surface cartilage or partial destruction of prominent points of bones; the joint exudate was clear or slightly clouded; proliferation of synovial tags and granulation tissue was the chief change. In advanced cases, however, there was tough fibrous thickening of the capsule, occasionally degeneration, calcareous, hyaline, or caseous; the joint exudate yellowish, greenish-yellow, with inspissated masses or detritus. The pus had no odor. The articular cartilages were destroyed in part and replaced by granulation tissue. Interosseous spaces were filled by

granulation tissue, in which plasma cell infiltration was marked. The plasma cells appeared reduced in number where polymorphonuclear, neutrophil leukocytes were present. The destruction of cartilage and bone occurred over opposing surfaces of joint heads and sockets, the points where mechanical pressure and friction were strongest, being most affected. In some cases many giant cells were present in the marrow, not only in Howship's lacunae, but generally near or in inflammatory areas. Occasionally new formation of bone tissue was noted near the exulcerated part of the cartilage or at the insertion of the capsule. Layers of osteoblasts were seen beneath the zone of provisional calcification. Where the cartilage was not destroyed, there was no regenerative process in the underlying bone.

Granulation tissue in some sections was found beneath the surface cartilage or even beneath epiphyseal cartilage, without denudation of the cartilage at those points.

In other cases the pathologic changes in the synovial membrane (synovitis) and the perichondrium (so-called pannus) seemed to constitute the primary process, and the osseous changes appeared to be secondary.

As the cases in which streptococci were discovered showed sinuses in the skin, the streptococcal infection might be considered as secondary.

In the few cases in which the tonsils were examined there were found in the tonsils peculiar pinhead sized yellowish, caseous points, sometimes as many as 20 being seen in the tissue of the tonsils, corresponding to the outlets of lacunae. These yellowish masses were not removed readily by forceps from the tonsillar parenchyma. Microscopically, they were small abscesses, surrounded by hyalin, or firm fibrous tissue. In some sections the parenchymatous cells of tonsils were almost entirely replaced by fibrous tissue. Smears and cultures showed hemolytic streptococci and various other bacteria, but no actinomyces. The relation of these streptococci to the joint inflammation will be discussed later.

The bacilli obtained may be described briefly as follows:

#### DESCRIPTION OF THE BACILLI

The Bacilli.—Rods of varying size depending on conditions and growth; on agar, length 1-1.5 $\mu$ , breadth 0.4-0.5; on serum, length 2-4 $\mu$ , breadth 0.4-0.5; sometimes in pairs and coccal forms; no capsule; nonmotile; no flagellae; gram-positive but some older cultures retain stain poorly; no odor in cultures either

aerobic or anaerobic; no visible growth on potato, but the implanted bacilli live 5 or 6 days; good growth on Loeffler's serum; no liquefaction (Culture 14 caused slight liquefaction in certain generations); in plain broth and ascites broth the supernatant fluid is sometimes clear, sometimes slightly opalescent with a small amount of whitish sediment; in dextrose broth there is slight diffuse cloudiness or the supernatant fluid is clear with sediment of yellowish brown tint; litmus milk is neither decolorized nor coagulated (Culture 14 decolorized milk); on gelatin a slight, whitish growth along needle track above its middle part, not at bottom; no liquefaction one week at room temperature. On plain agar growth is very poor in form of minute translucent round, flat colonies; condensation water almost clear, sometimes with small amount of whitish sediment; on blood agar the growth is good; nonhemolytic, only Cultures 10 and 19 showed slight hemolysis in certain generations; small round colonies after 24 hours, colorless, translucent, later whitish, but still semitranslucent.

Acid is produced in dextrose; in other sugars and higher alcohols acid production is variable. No indol is produced. The bacilli grow longer and more slender on Loeffler's serum, while on plain agar and blood agar they appear as very short bacillary or sometimes elongated coccal forms. In tissues the bacilli are usually short and frequently in coccal form, so that there is some difficulty in distinguishing them from diplococci. The bacilli show rather weak resistance to higher temperatures and lose transplantability on exposure of suspension in salt solution to 58 C. 15-30 minutes; one hour's incubation at 43 C. or half an hour at 55-56 C. does not prevent subsequent growth. The temperature optimum lies between 35-37 C.

Agglutination: In each blood agar tube of 24 hours' incubation 1 c.c. of physiologic salt solution was poured, and the colonies on the surface carefully rubbed off. This suspension was kept for 15 minutes at 58 C. and injected intravenously in rabbits at the intervals of 4-6 days. The doses injected of this suspension were increased in the order of 1-2-3 c.c. Eight days after the third injection the serum was used for agglutination tests. As rabbits are easily killed by living bacilli, heated suspensions were used. The results are illustrated in Table 1.

TABLE 1  
TITER OF AGLUTININ IN SERUM OF IMMUNIZED RABBITS

Serums	Cultures			
	11	16	19	20
11	320	160	320	160
19	320	320	640	160
20	40	80	40	80

Pathogenicity: The bacilli are variably pathogenic for guinea-pigs, mice and rats. Culture 11 did not kill guinea-pigs on subcutaneous injection of 0.3-5 c.c. of a blood agar culture suspended in 1 c.c. of salt solution, but killed mice in doses of 0.2-0.3 c.c., while Culture 5 killed guinea-pigs but did not kill mice in the respective doses. On the other hand, rabbits are undoubtedly susceptible. Intravenous injection of half or one blood agar slant of 24 hour cultures of Cultures 6, 11, 19, 20, all killed rabbits within 3-5-14 days. Marasmus and sepsis were the principal causes of death. Joint inflammation, chiefly synovitis, was demonstrated in two rabbits injected with Cultures 11 and 19. The result of subcutaneous injection is more variable. After death bacilli were found most frequently in the bone marrow.

In making injections the syringe, with needle attached, containing suspension of Culture 11, accidentally fell point down on the thigh of a man, perforating the clothing and skin. Next day a round, reddish, slightly infiltrated spot as large as a cent appeared; 2 days later the reddening spread over the anterior surface of the thigh with well defined, slightly elevated margin and burning pain. Temperature 99.5 F. lymph-node enlargement in the groin; in the course of a week the erysipelatous reddening extended over almost the entire anterior surface of the thigh, while at the point of inoculation in the center fading commenced. There was some edema and infiltration of the underlying tissue which subsided slowly under hot dressings. After two weeks all symptoms disappeared.

#### INOCULATION OF BACILLI IN SWINE

Fig 1.—Feb. 19, when 24 days old, one-fourth blood agar culture of Culture 11 suspended in salt solution was injected intravenously. No symptoms; edema of ear at site of injection.

May 26.—One blood agar slant of Culture 11 subcutaneously. No symptoms.

Apr. 12.—Two slants in 1 c.c. suspension subcutaneously.

Apr. 24.—Two slants in 2 c.c. solution intravenously.

May 3.—Two slants intravenously.

May 9.—Three slants intravenously.

May 16.—No lameness. Appears well.

Fig 3.—March 26, 59 days old, one blood agar slant of Culture 20 of bacillus, intravenously. No symptoms.

Apr. 6.—One slant intravenously.

Apr. 12.—One and one-half slants intravenously.

Apr. 18.—Right hind leg stiff.

Apr. 19.—Lame; quiet.

May 3.—Two slants of Culture 20 intravenously.

May 9.—Three slants intravenously.

May 16.—No lameness; appears well.

Fig 4.—March 26, 59 days old. Growth of Culture 14 of bacillus intravenously. No symptoms.

Apr. 12.—One and one-half slants intravenously; no symptoms.

Apr. 24.—Two slants intravenously.

Apr. 3.—Three slants intravenously.

May 11.—Pig unable to use left fore leg.

May 16.—Unable to stand. Left fore leg at shoulder swollen; animal looks sick.

May 20.—Left fore leg at elbow enormously swollen. Cannot stand at all.

May 28.—Killed. Large periarticular abscesses about elbow joint of left fore leg, communicating with joint cavity and containing thick yellowish odorless pus. Capsule thick, cartilage on joint head and socket destroyed and covered by granulations; shoulder and carpal joints normal. Elbow joint of right fore leg externally slightly swollen; circumscribed abscess with thick yellowish pus, involving lateral condyle of joint head and extending into the adjacent portion of the capsule; some capsular thickening; carpal joints externally normal and movable, but on dissection a small abscess is found in capsule without connection with joint cavity; cartilage normal; synovial fluid much increased, clear, colorless. In smears many gram-positive short bacilli in pus from the abscesses of right and left elbow joints and right carpal joint. Smears of spleen, epigastric and mesenteric lymph-nodes and bile showed the same bacilli. Heartblood, peritoneal fluid and urine free from bacteria. Inoculation of blood agar slants



with the pus of joints, the bile, lymph-nodes and spleen, gave minute nonhemolytic colorless colonies of the bacillus inoculated.

Fig 7.—May 3, 44 days old. Blood agar culture 24 hours old, of bacillary Culture 19, subcutaneously at right shoulder.

May 8.—Abscess at site of subcutaneous injection.

May 11.—Abscess about same. Right hind leg lame, swollen at knee.

May 14.—Swelling of right hind leg increased.

May 16.—Swelling increased; abscess in back same size as before.

May 22.—Killed. Abscess in back is about 5 cm. in diameter, filled with greenish yellowish pus with no odor; adjacent muscles pushed aside and partially destroyed. Right hind knee showed great thickening of capsule, edema in subcutaneous and muscular tissue; thick greenish-yellow pus in joint cavity; granulation tissue here and there on synovia; cartilage in places denuded; in several spots the granulation tissue extends into the bone. The tarsal joint shows some capsular thickening and a large periarticular abscess with greenish-yellow pus; the cartilage and bone of the joint destroyed in several places. Hip and phalangeal joints normal. Joints of left fore leg normal except that one phalangeal joint is swollen, due to a small abscess in the joint capsule with no apparent connection with joint cavity; cartilage and bone intact; synovial fluid clear, viscid.

Endocardium whitish, thick, not glistening; valves not deformed; no changes in lungs, spleen enlarged, hyperemic, no abscess. Liver and gallbladder normal. Kidneys normal; peritoneum rather opaque, fluid clear, not increased; slight fibrinous exudate over transverse colon and jejunum, mesenteric lymph-nodes rather large. Tonsils normal.

Many gram-positive short bacilli in smears from abscess in back, from right knee, tarsal and phalangeal joints; a few bacilli in smears from blood in heart, from spleen and mesenterial lymph-nodes. Smears from bile and renal pelvis negative. Minute, nonhemolytic dewdrop-like colonies developed in cultures from abscess in back, from left phalangeal, from right knees and tarsal joints, heart blood and spleen corresponding to culture originally inoculated.

Fig 8.—May 3, 44 days old. One blood agar slant of Culture 16, suspended in salt solution injected subcutaneously in back.

May 9.—Right fore leg lame. Induration at site of injection.

May 16.—No lameness; appears well.

June 19.—Killed. Right carpal joint showed proliferation of synovial tags and granulation tissue beneath capsule; cartilage denuded in one spot, and replaced by granulating fibrous tissue; synovial fluid clear, not much increased; capsule slightly thickened; other organs normal.

Fig 9.—Born about March 20, 1917. May 15, 56 days old. Suspension of one blood agar slant, Culture 11, subcutaneously. No symptoms.

Fig 10.—May 15, 56 days old. One blood agar slant, Culture 20, subcutaneously.

May 16.—Vomited; does not eat well.

May 17.—Well; no lameness.

Of 7 pigs, 3 were injected intravenously and 4 subcutaneously with bacilli. One of those injected intravenously showed temporary lameness in the hind leg (Fig 3), and one after repeated injections developed multiple arthritis resembling the natural disease (Fig 4). About the same results were obtained by subcutaneous injection—brief



lameness in one (Fig 8) and joint infection in another (Fig 7). Both methods of injection may cause joint infection in spite of the supposition that the intravenous method would seem the surer way, as observed in other pyogenic infections. Possibly subcutaneous wounds may be one point of entry of the bacilli in this form of arthritis in swine. In Fig 7 the joint suppuration developed some time after an abscess in the back had formed.

These experiments afford examples of different stages of arthritis, i. e., from synovial proliferation to bone degeneration, which correspond well to the conditions in the natural disease. The normal condition of the lungs and pleura differs notably from the descriptions in reports on swine arthritis caused by *Bacillus pyogenes*; in the latter pneumonia, lung abscess and pleuritis are described as frequent.

#### DESCRIPTION OF STREPTOCOCCI

Small cocci in pairs and short chains; no capsule; gram-positive. On plain agar slight growth; single colonies pinpoint in size; transparent after 24 hours, becoming more opaque later with clouding of water of condensation; no odor in cultures; no visible growth on potato; on Loeffler's serum good growth with liquefaction beginning in 24 hours at 37 C.; in plain and ascites broth supernatant fluid clear with a scanty white sediment; in dextrose broth, clear above with yellowish or brownish white sediment; litmus milk decolorized and acidified, coagulation in 48 hours, clot digested slowly in one case; in gelatin very slight whitish growth along needle track. No liquefaction after 6 days at 20 C.; on blood agar all cultures gave some hemolysis (weak in Cases 1 and 2, marked in Cases 3, 10, 13 and 21). The streptococci grow aerobically as well as anaerobically. No indol produced. Raffinose and mannite not fermented; inulin fermented by Cultures 1 and 3, slightly by 2, 10, 13, and 21; glucose, maltose, saccharose fermented; lactose fermented by 1 and 3, slightly by 2, 10, 13, and 21; salicin fermented by 1, 2, and 3, slightly by 10, 13, and 21.

TABLE 2  
FERMENTATIVE REACTIONS OF STREPTOCOCCI

Strains	Plain	Glucose	Inulin	Lactose	Maltose	Mannite	Neutral Red	Raffinose	Saccharose	Salicin
1	—	+	+	+	+	—	—	—	+	+
2	—	+	±	±	+	—	—	—	+	+
3	—	+	+	+	+	—	—	—	+	+
10	—	+	±	±	+	—	—	—	+	±
13	—	+	±	±	+	—	—	—	+	±
21	—	+	±	±	+	—	—	—	+	±

The streptococci obtained from the tonsillar abscesses gave very similar reactions.

With the serum of a rabbit injected with streptococcus, Culture 3, agglutination was obtained of Culture 3 at 2560, Culture 1 at 1280, Culture 2 at 80, Cultures 10 and 11 at 320, Culture 21 at 20, of streptococcus from tonsils of

Case 3 at 640, and of streptococci from other sources at 40-80. The streptococci were pathogenic for rabbits. As a rule, one 24-hour blood agar slant injected intravenously killed within 3 to 15 days from marasmus and sepsis; joint suppuration was a rather rare complication. Mice show variable susceptibility; rats relatively resistant.

#### INOCULATION OF STREPTOCOCCI IN SWINE

Fig 1.—April 6, when 70 days old, Fig 1 received one-half of a blood agar slant of *Streptococcus* 21 intravenously. No symptoms.

April 12.—One and one-half slants intravenously; no symptoms.

April 24.—Two slants intravenously.

May 3.—Two slants intravenously.

May 7.—Lame in right hind leg; no distinct swelling.

May 9.—Three slants intravenously; unable to stand.

May 10.—Better, but less active.

May 20.—Well.

Fig 5.—May 3, when 44 days old, received intravenously suspension of one blood agar slant of streptococci.

May 9.—Abscess appeared on left side of neck.

May 14.—Left hind leg lame.

May 16.—Abscess still present. Leg not distinctly lame.

May 21.—Abscess on neck, large as child's fist.

May 28.—Killed. In the left submaxillary region an abscess with fibrous edematous wall, containing thick yellowish pus with detritus. In left shoulder joint some synovial tags, fluid clear, not much increased. Left hock joint contained some proliferating synovial tags and some granulating fibrous tissue beneath the capsule; no destruction of cartilage or bone; fluid clear, viscid, slightly increased. Endocardium not glistening; some fibrous peritoneal adhesions. On cultures from abscess *Streptococcus hemolyticus*, *Staphylococcus aureus* and *B. subtilis* were obtained. Joint fluid negative.

Fig 6.—May 3, when 44 days old, received one slant of blood agar culture of *Streptococcus* intravenously.

May 8.—Right hind leg and left fore leg lame.

May 9.—Left fore leg swollen.

May 11.—Left fore leg more swollen, cannot run.

May 12.—Died. Extensive phlegmon in neck and left fore leg. In right hip joint granulation tissue beneath the capsule and in interosseal spaces; no denudation of cartilage; capsule fibrous. Hemolytic streptococci in smear and on cultures from phlegmon, heart-blood, spleen and hip joint. Cultures of phlegmonous part were contaminated by gram-positive and gram-negative large bacilli.

Altho there seems no doubt that the streptococci from swine arthritis can cause joint infection without the presence of the bacilli, it appears from a review of the pathologic changes in each case, that the streptococci as a rule are secondary to the bacillary joint infection. The streptococci may enter the body either through sinuses in the skin or through the tonsils. In fermentative reactions the streptococci obtained from joints resembled those from tonsillar abscesses in the corresponding cases.

The differences between swine streptococci and such bovine streptococci as we have been able to study seem to be that the former grow not so well anaerobically as aerobically; that the supernatant fluid in broth cultures of the former as a rule is clear; that they coagulate and acidify litmus milk, give more or less inulin fermentation, and are markedly pathogenic for rabbits.

#### SUMMARY

In 17 of 21 cases of chronic arthritis in swine, bacteria were found either in culture or in sections. In the 6 cases in which streptococci were found either alone or associated with bacilli, the lesions were of a chronic suppurative character, usually with external sinuses, and suggested in appearance chronic tuberculous lesions. However, careful search revealed no evidence of tuberculosis in sections, nor were there tuberculous lesions elsewhere in the animals from which these joints came.

In the 9 cases of pure bacillary infection, the joint changes varied in extent from early stages of a marginal fringe of granulation within the joint without destruction of cartilage to those of extensive exulceration of cartilage and disorganization and deformity of joints. In several of these bacillary cases, the joint fluid was clear or very slightly cloudy, with no gross evidence of suppuration. Examination of the aspirated joint fluid alone would in some instances have led to the conclusion that the joints were sterile, but bacilli were nevertheless present in sections and cultures from the granulation tissue.

The bacilli isolated from the joints in these cases are culturally alike, and in morphology and most cultural reactions resemble *B. pyogenes*, as described by Dutch and German veterinarians in cattle and swine, but differ from them in that they do not liquefy gelatin and Loeffler's serum. The pathologic changes produced by the bacillary infection resemble those ascribed to *B. pyogenes* in their chronic low grade suppurative character with tendency to the formation of granulation and fibrous tissue. The pulmonary infections described as frequent in animals infected with *B. pyogenes* were not observed in our cases, either in the natural disease or in experimental animals.

While the bacillary infection is apparently the etiologic agent in most of the cases included in this study, and the streptococcal infection apparently a later and secondary one, we do not wish to insist that infection by the bacilli described is the only cause of chronic arthritis

in swine. There may well be other organisms of suitable virulence which can set up similar processes. It seems important, however, to emphasize the element of nontuberculous infection in chronic deforming joint lesions in animals otherwise in good health.

The economic loss entailed by chronic arthritis in swine is considerable even in those animals which reach the market in otherwise good condition, and must be still greater among animals on infected stock farms. While much further study is necessary before a complete knowledge of the etiology of the disease is possible, the present study suggests some points which may be of value in preventing the disease in young pigs. Lesions of joints were produced experimentally by subcutaneous inoculation of bacilli as well as by intravenous. It is possible that puncture wounds of the skin in infected yards may be the means of initial infection in some instances. The alimentary tract cannot be excluded, for in one case we found a marked chronic nontuberculous infection of the mesenteric lymph nodes associated with arthritis. We were prevented from carrying out the feeding experiments planned by the entry of one of us in military service. We found no clear evidence of infection by way of the respiratory tract, although a chronic sinusitis was found in one hog. In the streptococcal cases, the streptococci isolated from the tonsils resembled culturally and immunologically those isolated from the joints.

The study of arthritic lesions in market hogs does not allow of conclusions concerning the relation of the arthritis to a possible more widespread infection of the original herds with *B. pyogenes*, in infections with which abscesses in various organs and muscles are said to occur. It has already been noted that the bacilli found in our cases differed from *B. pyogenes* in their failure to liquefy serum and gelatin. The joint lesions, however, correspond with those described in joint infections by *B. pyogenes*.

The occurrence of arthritis in some herds and not in others also suggests local sources of infection on the affected farms. It would seem advisable to exclude from the hog-lot any animals with chronic suppurations whether swine, cattle or other animals, for the same reasons that cattle and other animals suffering from tuberculosis are excluded to prevent tuberculosis among the hogs.

#### EXPLANATION OF PLATES 10 AND 11

Fig. 1 (Case 14).—Proliferation of synovial tags.  $\times 90$ .

Fig. 2 (Case 4).—Granulation tissue in place of synovial tags. 1. Plasma cell infiltration. 2. Dilated vessels. 3. Obliterated vessel.  $\times 70$ .

Fig. 3 (Case 9).—Destruction of surface cartilage. 1. Fibrous tissue in place of cartilage. 2. Island of cartilaginous tissue. 3. Bone tissue.  $\times 90$ .

Fig. 4 (Case 6).—Subchondral granulation tissue. 1. Proliferation of cartilaginous cells. 2. Zone of calcification. 3. Granulating fibrous tissue in marrow spaces. 4. Layer of osteoblasts.  $\times 60$ .

Fig. 5 (Case 21).—Giant cells in bone marrow. 1. Giant cells. 2. Granulating fibrous tissue in marrow spaces. 3. Bone trabeculae.  $\times 90$ .

Fig. 6 (Case 3).—Small abscesses in tonsils. 1. Abscesses. 2. Hyaline fibrous tissue.  $\times 60$ .

Fig. 7 (Case 6).—The bacilli in tissue, Gram stain.  $\times$  groups of bacilli.  $\times 1200$ .

Fig. 8 (Case 16).—The bacilli on blood agar culture, 24 hours.  $\times 1200$ .

Fig. 9 (Case 16).—The bacilli on Loeffler's serum; 24 hours.  $\times 1200$ .

Fig. 10.—Arthritis produced by inoculation of the bacilli from Case 19.



PLATE 10

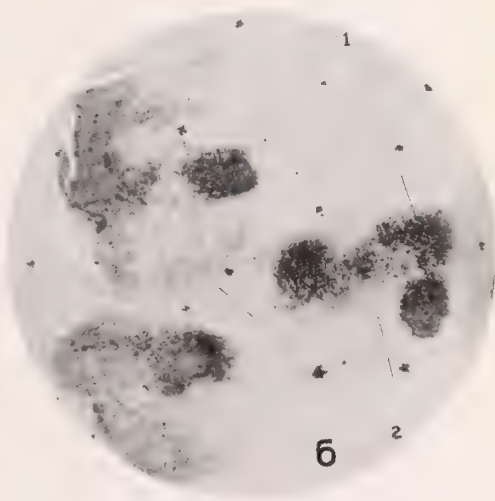
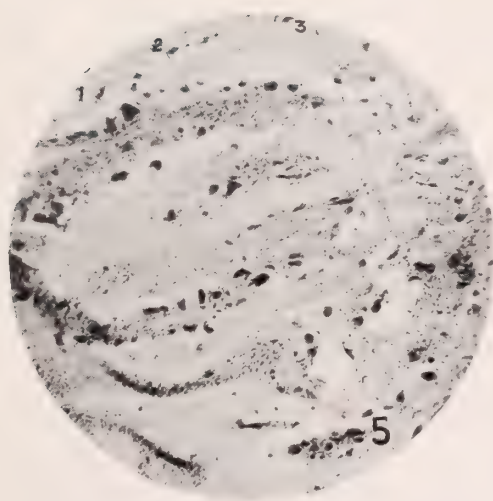
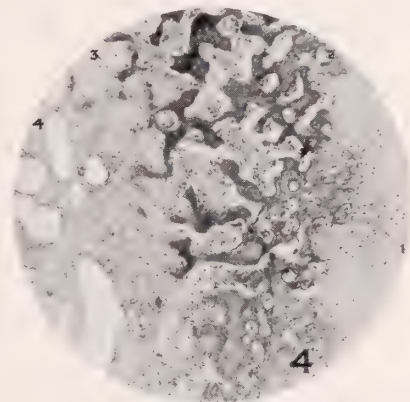
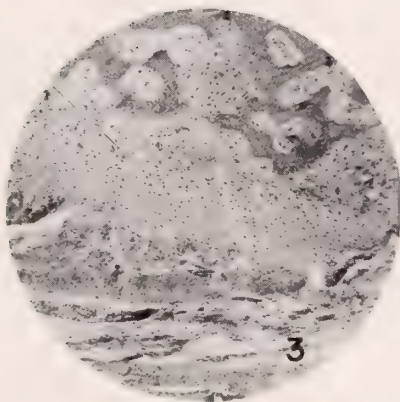
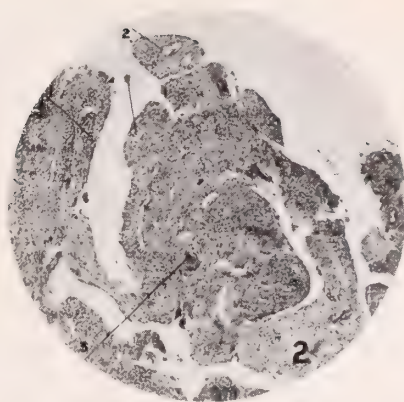
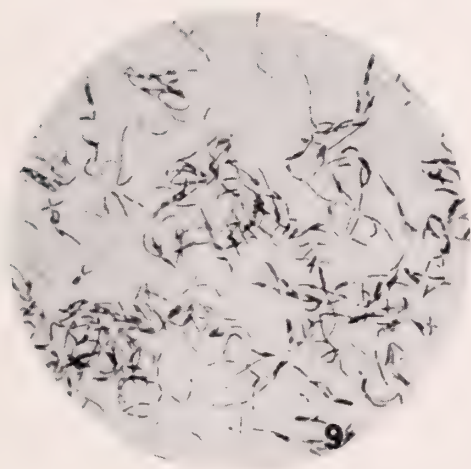
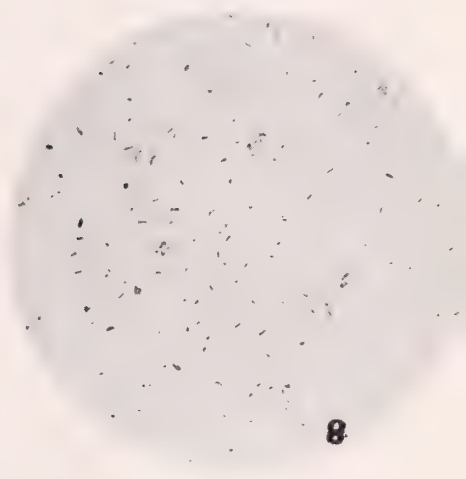
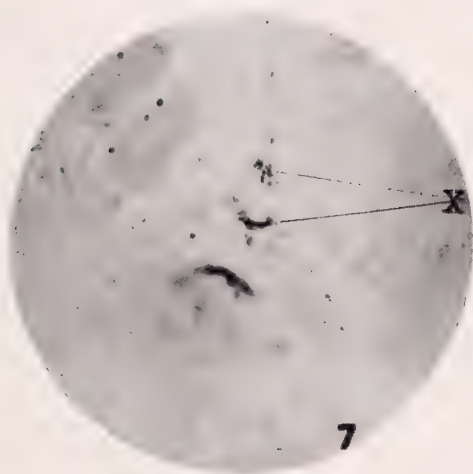




PLATE 11





# THE PATHOGENIC EFFECT AND NATURE OF A TOXIN PRODUCED BY *B. PARATYPHOSUS* B

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The fact that certain bacteria excrete substances which reproduce the symptoms caused by the organisms themselves, has led to the conclusion that bacteria, in general, produce disease by excreting poisons which are detrimental to a given host. Notwithstanding considerable evidence in favor of this conclusion, it has been possible in but few instances to isolate and identify toxic bacterial products as causes of pathologic effects.

This paper presents the results of experiments which indicate the production of soluble toxins by *B. paratyphosus* B, and make it probable that it is to these particular toxins that the bacillus of Achard and Bensaude owes its disease-producing power. Before entering on the discussion of the details of experimental data, a review of previous work is given.

Trautmann (1903),<sup>1</sup> Uhlenhuth (1906),<sup>2</sup> and others introduced the first suggestive evidence of the production of soluble toxins by *B. paratyphosus*. Rolly,<sup>3</sup> in 1906, made similar observations and determined a toxic action of 6 days old broth cultures. Cathcart,<sup>4</sup> in 1906, published evidence of thermostabile poisonous substances gained from autolyzed paratyphoid bacilli. Kraus and Stenitzer<sup>5</sup> (1907) communicated results which, although incomplete, showed that old alkaline broth cultures (11-27 days old) of *B. paratyphosus* contained certain substances which were fatal to the ordinary laboratory animals, producing diarrhea and paralysis of the posterior extremities. Unlike the toxin obtained by Cathcart, the poisonous substances of Kraus and Stenitzer were thermolabile. Franchetti<sup>6</sup> in the following year (1908) employing several strains, obtained inconstant results, but some toxins which he secured produced diarrhea, prostration, paralysis of the posterior limbs, and, in certain instances, coma and death on intravenous injections. In attempting to produce immunity to these substances in rabbits, Franchetti obtained a serum of such slight neutralizing action *in vitro* that he concluded it doubtful whether the serums of animals

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<sup>1</sup> Ztschr. f. Hyg. u. Infektionskrankh., 1903, 45, p. 139.

<sup>2</sup> Von Leuthold's Gedenkschrift, 1906, p. 1.

<sup>3</sup> Deutsch. Arch. f. klin. Med., 1906, 87, p. 595.

<sup>4</sup> Jour. Hyg., 1906, 6, p. 112.

<sup>5</sup> Wien. klin. Wchnschr., 1907, 20, p. 753.

<sup>6</sup> Ztschr. f. Hyg., u. Infektionskrankh., 1908, 60, p. 127.



thus immunized were protective against toxic filtrates. Konrich<sup>7</sup> in the same year (1908) offered also incomplete evidence of slight toxicity of sterile culture filtrates. The symptoms produced in mice were not characteristic, whereas guinea-pigs showed embarrassed breathing, chills, and prostration following intraperitoneal injections of the toxins. Yamanouchi,<sup>8</sup> in 1909, prepared highly toxic fluids by filtering 7-day broth cultures through Chamberland candles. His results have been criticized on the ground that his culture medium contained a high percentage of peptone, which is known to be toxic. He stated that typhoid toxin is neutralized by its homologous serum, while paratyphoid toxin is not. In contrast to the thermostability of the toxic filtrates noted by most observers, Yamanouchi found toxicity wholly destroyed at 100 C. Zwick and Weichel<sup>9</sup> (1910) were able to prepare a sterile toxic filtrate from a strain of *B. paratyphosus* isolated from a case of septic mastitis in cattle. This toxic filtrate produced acute symptoms of intoxication in mice and guinea-pigs. Hofmann<sup>10</sup> (1912) likewise obtained toxic-filtrates from paratyphoid bacilli. His filtrates retained their toxicity after heating for a full hour at 80 C. Schern<sup>11</sup> (1912) employing 4-week broth cultures passed through Berkefeld filters, found a low grade of toxicity, variable in pathogenic action and heat resistance. Messerschmidt<sup>12</sup> (1912) reported the presence of toxic compounds in filtrates of this organism, which he isolated from the stool of a patient with severe dysenteric symptoms.

In the work thus far cited, it will be observed that there is divergence of results as to the general properties of the poisonous substances obtained by the several workers, both as regards heat resistance and the production of symptoms. In addition to these more or less contradictory positive findings, there are negative results obtained by Brion and Kayser<sup>13</sup> (1902). Levy and Fornet<sup>14</sup> (1906) injected 48-hour broth culture filtrates into guinea-pigs and observed no toxic effects, although the culture used was virulent for guinea-pigs. Kutscher and Meinicke<sup>15</sup> (1906) recorded equally negative results from their experiments concerning the production of soluble toxins from Eberth Gaffky's bacillus and *B. paratyphosus*. Sacquépée<sup>16</sup> (1907) found the filtrates of *B. enteritidis* toxic and thermostabile, but not those of *B. paratyphosus*. Glaser<sup>17</sup> (1910) grew paratyphoid bacilli on meat, filtered the expressed juice through Reichel filters and injected heated and unheated filtrates into mice. The juice of the infected meat, incubated for 5 days at 37 C., and heated at 60 C., for 3 hours, killed mice in 18-24 hours. He concluded that the presence of a soluble toxin is doubtful. In the summary of his paper the statement was inserted that thermostabile toxins could not be demonstrated. Bidault<sup>18</sup> (1914) denied the existence of exotoxins in the paratyphoid-enteritidis group, and held that the toxic substance is firmly bound to the bodies of the organisms. During the food-poisoning epidemic, Bernstein and Fish<sup>19</sup> (1916) isolated a paratyphoid bacillus which failed to produce soluble toxins.

<sup>7</sup> Klin. Jahrb., 1908, 19, p. 247.

<sup>8</sup> Compt. rend. Soc. de biol., 1909, 66, p. 26.

<sup>9</sup> Arb. a. d. k. Gsndtsamte, 1910, 34, p. 391.

<sup>10</sup> Zur Kenntniss der Wirkung der Paratyphustoxine, Inaug. Diss., Heidelberg, 1912.

<sup>11</sup> Centralbl. f. Bakteriöl., I., O., 1912, 61, p. 15.

<sup>12</sup> Ibid., 1912, 66, p. 35.

<sup>13</sup> München. med. Wchnschr., 1902, 1, p. 611.

<sup>14</sup> Centralbl. f. Bakteriöl., 1906, 41, p. 161.

<sup>15</sup> Ztschr. f. Hyg. u. Infectiouskrankh., 1906, 52, p. 301.

<sup>16</sup> Compt. rend. Soc. de biol., 1907, 63, p. 328.

<sup>17</sup> Ztschr. f. Hyg., 1910, 67, p. 459.

<sup>18</sup> Compt. rend. Soc. de biol., 1914, 76, p. 422.

<sup>19</sup> Jour. Am. Med. Assn., 1916, 66, p. 167.

In view of the incompleteness and contradictory nature of previous work, I have made a systematic and comprehensive attempt to determine whether or not *B. paratyphosus* B may be induced to produce a true soluble toxin under suitable cultural conditions.

To this end I have employed largely a broth prepared as follows: 500 gm. of lean meat (beef) were covered with 1000 c.c. of water. This was macerated for 24 hours and then strained through wet cheese cloth, the juice expressed, and the original volume of the fluid restored by addition of water. The reaction was made neutral to phenolphthalein, and the broth inoculated with *B. coli* and incubated for 18 hours. The infusion was then sterilized in the autoclave. After filtration, 2% Witte's peptone was dissolved in the medium and the reaction determined. The range of the reaction was 0.3%-0.6% (normal acid). In early experiments the broth was made alkaline according to the method of Kraus and Stenitzer, namely by adding 2.4 c.c. of N/NaOH per liter of fluid previously made neutral to phenolphthalein. Witte's peptone always gave the most satisfactory results. The medium was then distributed in 1-liter flasks, each containing about 150 c.c., so as to keep the layer as shallow as possible. It was then sterilized for 10 minutes in the autoclave at 15 pounds, and inoculated with a standard loop from a 24-hour culture of bacilli in broth, and incubated at 37 C. After the incubation period, which was varied according to conditions, the culture was tested for purity and filtered through a Berkefeld N bougie. The sterility of the filtrates was determined by inoculation in agar, broth, and fermentation tubes.

Employing this medium, the toxin-production of Strain 2 (Appendix) was first tested. Under a variety of conditions, however, this strain was found to be devoid of all toxin-producing power in vitro. The organisms of this strain also possessed but slight virulence for mice.

Examination of the action of 9 additional strains of *B. paratyphosus* B, however, showed Strain 2 to be an exception rather than the rule. With all of the other strains the production of a toxic filtrate occurred. The degree of the toxicity of such filtrates and the effects on rabbits are shown in Table 1.

From Table 1 it appears that a toxic filtrate may be obtained from strains of *B. paratyphosus* B in general, when the organisms have been grown in a suitable broth for 8-14 days at 37 C., and that the toxicity is not that of the peptone constituent as such. Cultures in peptone-free mediums did not produce toxic substances. The controls show, however, that when upward of 5% peptone is used in the culture medium the toxicity of this substance becomes a complicating factor, as was undoubtedly the case in much previous work.<sup>8</sup> Strains 178, 180, and 185 gave the strongest toxic filtrates.

TABLE 1  
PRESENCE OF TOXIC SUBSTANCES IN 8-14 DAYS CULTURE FILTRATES

Number	Rabbit Weight, Gm.	Strain of Organism	Peptone % of the Broth	Age of Culture When Filtered, Days	Amount of Filtrate Injected, C.c.	Results
1	1115	12	1	14	5	Prostration 1 hr. and 15 min. after injection; marked dyspnea
2	1150	178	1	14	5	Prostration 1 hr. after injection. Marked dyspnea and paralysis of posterior extremities
3	1000	(Control)	1		5	No symptoms
4	850	178	1	14	3	Severe prostration 1 hr. after injection. Marked dyspnea and paralysis of hind limbs
5	850	96	1	14	3	Slight prostration. Slight dyspnea. No paralysis
6	800	(Control)	1		3	No symptoms
7	700	180	2	8	5	Prostration 1 hr. after injection. Marked dyspnea and paralysis. Death in 5 days
8	465	180	2	8	1	Prostration 1 hr. and 15 min. after injection. Marked dyspnea and paralysis. Death in 6 days
9	550	180	2	14	1	Prostration 45 min. after injection. Marked dyspnea and paralysis of posterior extremities. Death in 2 hr.
10	1030	180	2	14	5	Prostration 1 hr. after injection. Marked dyspnea and paralysis of posterior extremities. Death in 2 hr.
12	950	(Control)	2		5	No symptoms
43	1164	185	2	12	2	Prostration, severe dyspnea and paralysis of posterior extremities. Death 2 hr. and 15 min. after injection
44	859	185	2	15	2	Prostration 1 hr. after injection. Severe dyspnea and paralysis of posterior extremities. Death 12 hr. later.
45	664	(Control)	2		2	No symptoms
14	1350	178	3	14	5	Prostration 45 min. after injection. Severe dyspnea and paralysis of posterior extremities. Death in 2 hr.
15	1200	178	3	14	2	Prostration 45 min. after injection. Severe dyspnea and paralysis of posterior extremities
16	1000	(Control)	3		5	No symptoms
18	950	178	5	14	5	Prostration 45 min. after injection. Total paralysis. Severe dyspnea. Convulsions. Death in 1 hr. and 45 min.
19	1200	178	5	14	5	Prostration 45 min. after injection. Severe dyspnea and complete paralysis. Death in 4½ hr.
20	1000	178	5	14	2	Prostration 1 hr. after injection. Severe dyspnea and complete paralysis. Death in 5¼ hr.
21	1000	(Control)	5		5	Prostration; death in 18 hr.

Having shown that *B. paratyphous B* produces substances which are toxic when introduced into the circulating blood, I next determined the relative toxicity of such filtrates when introduced intraperitoneally and subcutaneously. In general the toxic action was relatively slight following injection by the latter avenues, as illustrated in Table 2.

TABLE 2

COMPARATIVE TOXICITY OF FILTRATES INTRODUCED INTRAVENOUSLY, INTRAPERITONEALLY, AND SUBCUTANEOUSLY

Number	Weight Rabbit, Gm.	Toxin 178 12 Days Old	Amount Injected, C.c.	Route	Results
25	580	Filtrate, 12 days old	2	Intravenous	Marked prostration, diarrhea, dyspnea, and paralysis; death in 48 hr.
23	960	Filtrate, 12 days old	5	Intraperitoneal	Slight prostration 3 hr. after the injection
24	950	Filtrate, 12 days old	5	Subcutaneous	Normal
28	900	Control broth	5	Intravenous	Normal
27	650	Control broth	5	Intraperitoneal	Normal

Table 2 shows that the same filtrate which produces marked symptoms of intoxication when injected intravenously has little or no effect when introduced intraperitoneally or subcutaneously.

The results thus far discussed were those obtained by the toxic filtrates from cultures of considerable age, namely, 8-14 days. To determine whether or not the toxicity was dependent on the products of bacterial autolysis, a number of series of tests was made beginning with relatively young cultures, 24 hours old, and ending with 15-day cultures. A total count of the viable cells was made after 24 hours' growth, and also before filtration of the cultures contained in each flask.

When injected into rabbits the filtrates from 24-hour cultures of Strains 180 and 185 also caused symptoms of intoxication which were the same as those produced by the filtrates of older cultures (Tables 3 and 4).

Tables 3 and 4 show that filtrates from 24-hour cultures of Strains 180 and 185 possessed a distinct lethal action on rabbits, the symptoms of intoxication being similar to those produced by the filtrates from cultures a week or more old. Further tests bearing on the same point were made by employing all the other strains (Appendix). In all these instances, a potent toxin could be obtained in young cultures, but the toxicity of the filtrates varied greatly with the different strains. The alkalinity of the filtrates progressively increased with the age of the cultures. The plain broth was without effect on the animal.

Having thus established the fact that certain strains of *B. paratyphosus* B actually produce toxic-broth filtrates, both in young and



TABLE 3

TOXIC ACTION OF FILTRATES FROM 1-, 3-, 6-, 9-, 12-, AND 15-DAY CULTURES OF STRAIN 180,  
B. PARATYPHOSUS B.

Number	Rabbit Weight, Gm.	Reaction of Culture Medium + Acid - Alkaline	Age of Culture at Filtration	Number of Organisms per C.c. of Cultures	Number of Non-viable Cells per C.c.	Amount Injected Intravenously, C.c.	Results
29	550	+0.1	24 hr.	2870000000		3	Severe prostration, dyspnea, diarrhea, paralysis of hind limbs and spasms; death in 1 hr. and 40 min. after injection
30	1110	+0.1	24 hr.	2870000000		2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; death in 1 hr. and 45 min.
31	510	+0.1	24 hr.	2870000000		0.5	Prostration, dyspnea, recovery
32	1300	-0.3	72 hr.	4570000000	3659000000	2	Severe prostration, dyspnea, and paralysis of hind limbs; death 36 hr. after injection
33	1100	-0.6	24 hr. 6 days	6100000000 3240000000	5776000000	2	Severe prostration, dyspnea, and paralysis of hind limbs; death 10 days after injection
34	1200	-0.8	24 hr. 9 days	4600000000 1160000000	3440000000	2	Prostration, dyspnea, slight diarrhea, but no paralysis; death 12 hr. after injection
35	1300	-1.3	24 hr. 12 days	6100000000 6150000000	5485000000	2	Slight prostration; recovery
36	1080	-1.0	24 hr. 15 days	3980000000 700000000	3280000000	2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; convulsions and death in 2 hr. and 15 min.
37	1650	-1.0	24 hr. 15 days	3980000000 700000000	3280000000	4	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; convulsions and death 12 hr. after injection
38	1610	+0.3 control broth				4	Not sick

TABLE 4

TOXIC ACTION OF FILTRATES FROM 1-, 3-, 6-, 9-, 12-, AND 15-DAY OLD CULTURES OF  
STRAIN 185, B. PARATYPHOSUS B.

Number	Rabbit Weight, Gm.	Reaction of Culture Medium at Filtration + Acid - Alkaline	Age of Culture at Filtration	Number of Organisms per C.c. of Cultures	Number of Non-viable Cells per C.c.	Amount Injected Intravenously, C.c.	Results
39	760	+0.25	24 hr.	5200000000		2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; death 12 hr. after injection
40	700	-0.3	24 hr. 72 hr.	5100000000 2100000000	3000000000	2	Prostration, dyspnea; no diarrhea or paralysis; recovery
41	660	-0.4	24 hr. 6 days	7200000000 2200000000	5000000000	2	Prostration, dyspnea; no diarrhea or paralysis; recovery
42	757	-0.8	24 hr. 9 days	1100000000 1410000000	9590000000	2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; recovery
43	1164	-0.9	24 hr. 12 days	2800000000 1270000000	1530000000	2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; death in 1 hr. and 45 min. after injection
44	850	-1.0	24 hr. 15 days	1000000000 290000000	9710000000	2	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; convulsions prior to death; death in 8 hr.
45	664	+0.4 control broth				2	Not sick



old cultures, I next attempted to determine whether or not the substances conferring this toxicity were true in the sense that they were susceptible to specific antitoxin neutralization. To this end, the toxic filtrates were injected into rabbits and the serums, tested from time to time for their power of elimination of the toxic action of such filtrates, when injected intravenously into normal rabbits. Table 5 shows the action of a neutralizing serum thus obtained. The serum tested in this experiment was that of Immune Rabbit B which had been subjected to the injection of 21 c.c. of the toxic filtrates from 24-hour cultures of *B. paratyphosus* B Strain 185. The 1st injections were made subcutaneously (2 injections of 5 c.c.); an intermediate injection (intraperitoneally of 5 c.c.), and finally 3 doses intravenously (2 c.c.). The whole period of immunization comprised 37 days.

TABLE 5

ANTITOXIC ACTION OF SERUM OF RABBIT IMMUNIZED WITH TOXIC FILTRATES OF STRAIN 185, *B. PARATYPHOSUS* B \*

Number	Rabbit Weight, Gm.	Amount of 24-Hr. Filtrate from Strain 185 Employed as Toxin, C.c.	Amount of Serum Used for Neutralization of Toxin, C.c.	Results
63	500	2	.....	Death in 5½ hr. after injection
64	600	2	2, normal	Death 2 hr. after injection
65	650	2	1, immune B	Death 3 days after injection
66	615	2	2, immune B	Slightly sick; recovery
67	700	2	3, immune B	No effects
68	725	4	4, immune B	Slightly sick; recovery .

\* Serum-toxin mixtures allowed to stand 1 hr., at 37 C., and then injected intravenously into normal rabbits.

The experiments in Table 5 show that the toxicity of the broth filtrate is due to a content of true toxin, inasmuch as the injection of such filtrates into a suitable host induces the production of specific neutralizing substances not present in normal serum.

Further evidence of the toxin-neutralizing power of Immune Serum B is given in Table 6. Native and boiled toxins were used in the experiments described.

Table 6 clearly indicates inhibition of the toxic action of the filtrate by Immune Serum B, whether the filtrate has been heated or not.

A similar experiment was conducted with the toxin of Strain 180, *B. paratyphosus* B and its specific antiserum. For this purpose Immune Rabbit C received 2 subcutaneous injections of 5 c.c. each of

TABLE 6

ANTITOXIC ACTION OF SERUM OF RABBIT IMMUNIZED WITH TOXIC FILTRATES OF STRAIN 185, *B. PARATYPHOSUS B* \*

Number	Rabbit Weight, Gm.	Amount of 24-Hr. Filtrate from Strain 185 Employed, C.c.	Amount of Serum Used for Neutralization of the Toxin, C.c.	Results
70	1050	2 (5 min. at 100 C.)	.....	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; death in 12 hr.
71	900	2	2.5, normal	Severe prostration, dyspnea, diarrhea, and paralysis of hind limbs; death in 8 hr.
72	850	2	2.5, immune B	No symptoms
73	940	2 (5 min. at 100 C.)	2.5, immune B	No symptoms

\* Serum-toxin mixtures allowed to stand 1 hr., at 37 C., and then injected intravenously into normal rabbits.

TABLE 7

ANTITOXIC ACTION OF SERUM OF RABBIT C IMMUNIZED WITH TOXIC FILTRATES OF STRAIN 180, *B. PARATYPHOSUS B* \*

Number	Rabbit Weight, Gm.	Amount of 24-Hr. Filtrate from Strain 180 Employed, C.c.	Amount of Serum Used for Neutralization of Toxin, C.c.	Results
74	780	2	.....	Severe prostration, dyspnea, diarrhea, and paralysis of posterior extremities; recovery
75	560	2	2, normal	Severe prostration, dyspnea; diarrhea, and paralysis of posterior extremities; recovery
76	530	2	1, immune C	Slight prostration, dyspnea
77	525	2	2, immune C	Slight dyspnea
78	705	4	4, immune C	Slight prostration, dyspnea, and diarrhea; recovery

\* Serum-toxin mixtures allowed to stand 1 hr., at 37 C., and then injected intravenously into normal rabbits.

this toxin; 10 c.c. intraperitoneally, and 2 of 2 c.c. intravenously over a period of 37 days. The toxin employed in this experiment failed to kill the control rabbits but produced an acute and severe toxemia (Table 7).

The results recorded in Table 7 show a definite elimination of the symptoms of the toxemia through the action of Immune Serum C.

Further evidence of the antigenic power of the culture filtrates is seen in the fact that the immune serums possess a marked specific agglutinating action for *B. paratyphosus B*. Thus the serum of Immune Rabbit B and of Immune Rabbit C discussed above; in addition to their antitoxic action, agglutinated suspended paratyphoid organisms in a dilution of 1:1000 and 1:1100, respectively. The

serums also produced complete complement deviation in the dilution 1:1000.

It appears established, therefore, that sterile toxic filtrates of B. paratyphosus B contain substances which stimulate the formation of specific antibodies, among which is an antitoxin.

Certain additional observations concerning an active immunity established in a goat and the stability, precipitability, and dialysis of the toxin, and effects on animals are here recorded.

*Active Immunity in Goat.*—A goat actively immunized against 24-hour culture filtrates of Strain 185, B. paratyphosus B, received in all 738 c.c. of the toxin during a period of 3 months. The injections were given in the subcutaneous tissues in increasing doses starting with 5 c.c. and ending with 75 c.c. At first the animal displayed marked prostration manifested by stupor, chills, and fever. An increase in temperature of 4.6 C. was recorded 4 hours after the 1st injection was made. The subsequent injections were followed by an increase of 2-3 degrees in temperature. Toward the end of the treatment the animal tolerated 75 c.c. at 1 injection without the slightest symptoms of prostration. The serum of this animal agglutinated B. paratyphosus B 185 suspended in NaCl solution in a dilution of 1:200. Furthermore, the serum protected normal rabbits against the toxic effects of 24-hour culture filtrates of B. paratyphosus B 185 in as low a quantity as one half of 1 c.c. against the usual 2 c.c. dose of the toxin. Control tests with normal goat serums showed that they do not possess this neutralizing property.

*Stability of Toxin as to Time.*—When stored in the refrigerator at zero or at 3-4 C. in a full, sealed receptacle, the filtrate retained an undiminished toxicity for a period of 4 months.

*Thermostability.*—Boiling the toxin at 100 C. for 5 minutes or heating at 80 C. for 15 minutes does not appreciably diminish its toxicity. Even heating for 5 minutes in the autoclave, at 15 pounds, has not completely destroyed the toxin of Strain M. The results of some of these experiments are given in Table 8.

We may safely say that the toxin of B. paratyphosus B is relatively thermostable.

*Precipitation of the Toxin.*—The toxic bodies produced by these bacilli are precipitated by absolute alcohol and ammonium sulfate. The dried alcoholic precipitate redissolved in salt solution 0.85% manifests in rabbits the usual symptoms of toxemia, whereas the residue of the filtrate after evaporation in vacuo does not produce a state of toxemia.

*Dialysis of the Toxin.*—A very strong toxin prepared from Strain 180 after 24 hours' growth was dialyzed under aseptic conditions for 3 days in a collodion sac. The dialysate was injected in 4 c.c. doses into the ear vein of a rabbit without the slightest effect, while 2 c.c. of the toxin killed the control rabbit within 2 hours. The same experiments were repeated with the toxin of Strain 185, with the same results. The toxin itself retained its full strength during the dialyzing period. This experiment shows that the toxin is non-dialyzable.

*Effect of the Toxin on Rabbits, Guinea-Pigs, Cats, and Mice.*—It may be stated, as a general rule, that when paratyphoid toxin is injected into the ear

TABLE 8  
EFFECT OF HEAT ON TOXIC CULTURE FILTRATES *B. PARATYPHOSUS B*

Num- ber	Rabbit Weight, Gm.	Strain of Organism	Age of Culture at Filtration	Degree of Heat Applied	Amount Injected Intra- ven- ously, C.c.	Results
53	420	C	24 hr.	.....	2	Marked prostration, dyspnea, diarrhea, and paralysis of posterior extremities; recovery
54	460	C	24 hr.	.....	2	Marked prostration, dyspnea, diarrhea, and paralysis of posterior extremities; recovery
55	500	C	24 hr.	5 min. at 100 C.	2	Prostration, dyspnea, and paralysis of posterior extremities; recovery
56	500	M	24 hr.	.....	2	Severe prostration, dyspnea, diarrhea, and paralysis of posterior extremities; recovery
57	500	M	24 hr.	5 min. at 100 C.	2	Severe prostration, dyspnea, diarrhea, and paralysis of posterior extremities; recovery
58	550	M	24 hr.	5 min. at 15 lb., in autoclave	2	Prostration, dyspnea, and paralysis; less marked when compared with 56 and 57
43	1164	185	12 days	.....	2	Severe prostration, dyspnea, diarrhea, and paralysis of posterior extremities; death in 1 hr. and 45 min.
47	1033	185	12 days	40 min. at 56 C.	2	Severe prostration, etc.; recovery
48	765	185	12 days	15 min. at 80 C.	2	Prostration, dyspnea, and paralysis less marked; severe diarrhea; recovery
49	700	185	12 days	5 min. at 100 C.	2	Prostration, dyspnea, severe diarrhea, and paralysis of hind limbs; death in 2 hr.

vein of a rabbit in doses of 2 c.c. no symptoms whatever appear in less than 45 minutes following the administration of the poison. Soon after the injection is made the animals seek seclusion, turn restlessly, and show embarrassed breathing. This dyspnea increases gradually; the animal in most cases tries to keep its head raised. There is definite lack of coordination. If pushed, it falls on its side and does not attempt to change its position. In severe cases, partial paralysis follows, especially marked in the posterior extremities. This stage may last for 2 hours, and usually ends in death. Very strong toxins kill in 2 hours in doses varying from 1-2 c.c. In severe toxemias violent convulsions have been observed prior to death. The animals in this condition relapse quickly into the comatose state. I have not observed recovery in any rabbit which reached this convulsive stage. In a great majority of the intoxicated animals, the symptoms are accompanied by a severe diarrhea with an excessive amount of fluid, or by discharge of fecal balls. Animals passing through this diarrheal stage usually have a good chance for recovery. It was also noticed that the heated toxins produce in some instances a much more severe diarrhea than the unheated ones. Very often the animals surviving the immediate severe attack are found dead the next morning. If death does not occur within 24 hours, the animals are likely to survive. Blood examinations made from the intoxicated rabbits revealed a leukopenia and also a decrease of hemoglobin, possibly due to a laking of erythrocytes. Injections of this toxin seem to increase the peristalsis markedly. From a series of 4 experiments, each with boiled and unboiled toxins in amounts of 1-2 c.c., it was shown



that this particular toxin has no direct effect on the contractions of muscle strips from the pyloric end of the stomach greater-curvature. Pilocarpin and atropin were used in the control sets. Practically all the animals surviving the toxemia became greatly emaciated.

After death congestion of the liver, spleen, intestine, and lungs were usually present. Parenchymatous degeneration of the liver was common. The intestine and peritoneal cavity contained a great amount of fluid. Hemorrhages were often found in the lungs. Paralysis of the bladder was also noted. The other viscera do not as a rule exhibit any other definitely marked gross changes.

Intraperitoneal injections of 5 c.c. of a powerful 24-hour filtrate of Strain 185 into 2 guinea-pigs controlled by intraperitoneal injection of pure broth into another guinea-pig resulted in great loss of weight of the first 2 animals. Following the injection both pigs appeared prostrated and were very quiet. By the next day 1 of the pigs had lost 40 gm. and the other, 50 gm. in weight. The original weight was, however, quickly restored. The 3rd animal remained well.

Two cats, each weighing about 900 gm., were given subcutaneous injections of a 14-day broth culture filtrate from Strain 178 in 10 c.c. dosage. The broth contained 3% peptone and was alkaline to phenolphthalein. A 3rd cat, also about the same weight, received the broth alone. Only 1 cat was slightly affected. This was the cat that received an injection of the toxin. The animal appeared sick on the next day, but recovered quickly. The other cat, which received the toxin, was in good condition. A 4th cat, about 1500 gm., was fed with meat infected with Strain 185, without effect. A 5th cat, weighing about 700 gm., received boiled infected meat (Strain 185) also without any signs of poisoning.

Following the subcutaneous injection of paratyphoid B toxins, the mice became prostrated, but most of them recovered. Intraperitoneal injections often leads to death.

#### SUMMARY

The evidence presented by early workers points in the direction of possible endotoxins as the cause of the pathogenic effects of *B. paratyphosus* B; while my own results indicate the presence of soluble toxins. It is possible that both exotoxins and endotoxins are produced by these bacilli. It is to be emphasized that the soluble toxins obtained are of low potency, if compared with the toxins of *B. diphtheriae* or *B. tetani*. Another important distinction between these types is the relative thermostability of this particular bacterial poison. The production of this toxin is a matter of great difficulty, owing to the variations of the organisms, growth conditions, and susceptible hosts. The general effects of the filtrates on rabbits following intravenous injections are essentially the same as those observed by Franchetti, Kraus and Stenitzer, Yamanouchi, and others. The toxemia strongly resembles an anaphylactic shock. Franchetti attempted to produce an antitoxic serum, and found that the serum of animals



immunized against either extracts or filtrates are only in certain dosages protective. Indeed, Franchetti concluded that it is doubtful whether the serum of animals immunized against filtrates will protect against the toxic filtrates. The serum of rabbits immunized against the filtrates protected other rabbits against the toxic extracts in proportion of one to one. If a stronger extract was used, the effect was nil. Franchetti's antitoxic serum possessed agglutinating properties and also some bacteriolysins. Kraus and Stenitzer reported that antitoxins produced to the toxins of *B. typhosus* protected the experimental animals not only against the typhoid toxin but also against paratyphoid toxin. These authors express the opinion that both typhoid and paratyphoid toxins might be bodies with antigenic properties. In a recent article, Smith and Ten Broeck<sup>20</sup> report the discovery of a toxin produced by the bacillus of fowl typhoid, which seems to be closely related to the toxin produced by *B. paratyphosus* B. The authors believe that their toxin is probably an endotoxin, for increased resistance toward it can be produced only with great difficulty and to a relatively slight degree. They further state that the resistance of animals immunized against toxic filtrates suggests more a state of tolerance than true immunity. Furthermore, their experiments designed to demonstrate the presence of antibodies in the blood of treated rabbits have indicated some toxin-inhibiting power, but this was far below what might have been expected in case of a true exotoxin.

The assumption seems justified that toxic compounds are present in young filtered broth cultures of *B. paratyphosus* B, and the immunity experiments tend to show that the toxins produced by these bacilli are bodies with antigenic properties.

#### CONCLUSIONS

In broth cultures soluble toxic substances are produced within 24 hours by some strains of *B. paratyphosus* B.

These toxic substances produce constant pathologic effects and are of the nature of true soluble toxins, inasmuch as they stimulate the formation of antitoxins.

The toxic substances are comparatively themostabile, since they resist boiling for 5 minutes at 100 C.

<sup>20</sup> Jour. Med. Research, 1915, 31, p. 523.

## APPENDIX

STRAIN 4.—From the American Museum of Natural History. The organism is apparently avirulent and does not produce toxins.

STRAIN 12.—Isolated from blood in paratyphoid fever October, 1912.<sup>21</sup> The organism is virulent (when subcutaneously injected); 0.1 of 1 c.c. of a 24-hour broth culture kills a white mouse in 14-19 hours. The toxin-generating power of this strain is not very high.

STRAIN X.—From the Department of Health of the City of New York, through the courtesy of Dr. W. H. Park. It is not virulent and produces only weak toxins.

STRAIN Y.—Origin unknown but believed to have been isolated at the Army Medical School by Major Russell, about 1908. The bacillus, although not very virulent, produces strong toxins in broth cultures.

STRAIN 180.—Isolated from pie mixture in a food-poisoning outbreak at Westerly, R. I., by Dr. H. Bernstein.<sup>22</sup> The organism is a very good toxin-producer, but not highly virulent. One tenth of 1 c.c. of a 24-hour broth culture kills a mouse in 4 days when subcutaneously injected.

STRAIN 185.—Isolated by Dr. Cole from the pus of a suppurating lymph gland in the neck of a patient.<sup>23</sup> The bacillus produces strong soluble toxins, but is not very virulent.

STRAIN 209.—Isolated August, 1916, from human blood. The organism is virulent. The usual dose kills a mouse within 24 hours. Its toxigenic power is not very marked.

STRAIN 210.—Isolated from human blood, August, 1916. The organism is virulent. One tenth of 1 c.c. kills a mouse within 24 hours.. It has a moderate toxin-producing power.

STRAIN 211.—Obtained from feces of a paratyphoid case, August, 1916. The toxin-producing power of this strain is decidedly higher than of Strains 209 or 210.

With the exception of Strains X and Y, these cultures were obtained from Professor Jordan. Full data as to the characteristics of these strains are given in his article.<sup>24</sup>

<sup>21</sup> Irons and Jordan: Jour. Infect. Dis., 1915, 17, p. 234.

<sup>22</sup> Jour. Am. Med. Assn., 1916, p. 167.

<sup>23</sup> Jour. Infect. Dis., 1916, 18, p. 349.

<sup>24</sup> Ibid., 1917, 20, p. 457.

# DIFFERENTIATION OF THE PARATYPHOID- ENTERITIDIS GROUP, II

## LEAD ACETATE AGAR

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Variations in rapidity and intensity of hydrogen-sulfid production have been suggested by several observers as a means of bacterial differentiation. Orłowski<sup>1</sup> found that *B. typhosus* produced a black precipitate, lead sulfid, in lead acetate medium, while *B. coli* did not. Sacquépée and Chevrel<sup>2</sup> confirmed these results and showed further that a culture of *B. paratyphosus* B blackened lead acetate medium even earlier than did *B. typhosus*, while one of *B. paratyphosus* A was negative, thus resembling *B. coli*. Burnet and Weissenbach,<sup>3</sup> applying this method to 517 cultures isolated in the French army during the present war, found that the results obtained with lead acetate medium corresponded in all cases with the results of the agglutination tests. *B. paratyphosus* B blackens the medium in 18 hours; *B. typhosus* blackens the medium a little more slowly and less intensely; *B. paratyphosus* A grows either without blackening at all or only after several days. Hollande and Beauverie<sup>4</sup> advocate the use of test papers of various sorts, among them one with lead acetate, for the differentiation of this group, and report results essentially the same as those of earlier authors.

Strains of *B. enteritidis* and *B. suis* *typhimurium* do not seem to have been tested by any of the investigators, and no comparative series of cultures with definitely ascertained characters has been worked out. For these reasons, we have used a lead acetate medium for comparing the behavior of 74 strains already studied in considerable detail by Jordan.<sup>5</sup>

Preliminary tests showed the most favorable medium and mode of procedure to be as follows:

Three per cent. Witte's peptone was dissolved (by boiling) in fresh meat broth (1 pound lean beef to 1 liter water). After filtering this broth, 1.5% agar was dissolved in it, the reaction brought to 1% acid on the phenolphthalein scale, and the medium tubed and sterilized. Then the tubes were cooled to 43 C.,

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<sup>1</sup> Beitrag zur Kenntniss der biologischen und pathogenen Eigenschaften der *Bacterium coli communis*. Diss., St. Petersburg, 1897.

<sup>2</sup> Compt. rend. Soc. de biol., 1905, 59, p. 535.

<sup>3</sup> Ibid., 1915, 78, p. 565.

<sup>4</sup> Ibid., p. 722.

<sup>5</sup> Jour. Infect. Dis., 1917, 20, p. 457.

and 2 drops (0.1 c.c.) of a 10% lead acetate solution made from recently sterilized water were added to each tube and the tube well shaken. On cooling, the tubes were inoculated by sliding the needle in between the agar and the wall of the tube (duplicates for each organism). These tubes were then incubated at 37 C. for 18-24 hours.

A positive reaction is indicated by a blackening of the medium along the needle track, often spreading over the whole surface. This is generally apparent in 6-8 hours, but the sharpest results are obtained in 18-24 hours.

The 74 strains tested in the lead acetate agar have given the following results:

20 strains of <i>B. paratyphosus</i> A.....	Negative
28 strains of <i>B. paratyphosus</i> B.....	Positive
20 strains of <i>B. suipestifer</i> .....	Negative
6 strains of <i>B. enteritidis</i> .....	Positive

These are the same strains described in detail in an earlier paper.<sup>5</sup>

The hydrogen sulfid reaction shows an exact correspondence with the agglutination and fermentation reactions there described. Some slight irregularities are occasionally observed, as in the case of the fermentation reactions, but the principal group distinctions are quite as sharp as those based on other characteristics. It must be especially noted that the 5 strains of the *B. paratyphosus* type, which are of porcine origin (Nos. 62, 115, 161, 169 and 175, for the full description and history of which see Footnote 5), have shown more irregularity than any other strains, occasionally yielding a negative reaction when plated and subcultures from individual colonies are tested. Thus in 1 instance, 1 colony of 10 of No. 169 gave a negative reaction. The variability of these strains corresponds with the variability which they have shown in other respects, and which has been recorded in the first paper. It is perhaps significant that the 5 strains of *B. paratyphosus* B, which are of porcine origin, should in this respect show some affinity to the *B. suipestifer* type.

#### SUMMARY

In lead acetate agar, all typical paratyphoid A strains (20) fail to blacken the medium in 18-24 hours. All strains of *B. enteritidis* (6) give a positive reaction. The great majority of *B. paratyphosus* B strains (23 of 28) give a consistently positive reaction while all *B. suipestifer* strains (20) are negative. Five strains of porcine origin, belonging to the *B. paratyphosus* B type, are not constant in their reactions, but these are the same strains that in Jordan's earlier study have been found variable and irregular in other respects.

# THE HYDROGEN-ION CONCENTRATION OF CEREBROSPINAL FLUID

## STUDIES IN MENINGITIS. I

A. LEVINSON

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In the course of our investigation of meningitis we found it necessary to study the physicochemical conditions of the spinal fluid. We began this phase of our work with a study of the hydrogen-ion concentration of the fluid. In a previous investigation<sup>1</sup> in which we used methyl red as an indicator, we found that all spinal fluid was alkaline to methyl red, and that the titrable acidity of the fluid varied with different conditions, the fluid of tuberculous meningitis showing the same acidity, or, as we then termed it, the same alkalinity as the normal fluid, and the fluid of epidemic meningitis showing a higher acidity than the normal. We soon discovered, however, that the fluid on which we were working, had been standing too long before examination to give an accurate idea of the acidity. Besides, we were also aware of the fact that the titrable acidity did not represent the true hydrogen-ion concentration of the fluid. We therefore decided to determine whether a similar variation in the free H-ion concentration occurs in different conditions. With this in view, we examined both meningitic and nonmeningitic fluids.

### METHOD

At first we made use of the gas-chain method described by Michaelis<sup>2</sup> and the one suggested by Hildebrandt<sup>3</sup> for the determination of the H-ion concentration of spinal fluid. Very early in our work, however, we discovered that acidity measured by the gas-chain method was considerably lower than that determined by the indicator method against standard solutions. This discrepancy was found to be due not to any fault of either method, but to the variations occurring in the H-ion concentration of fluid that had been standing even one-half hour, the time it takes to reach the laboratory and put the gas-chain apparatus in working order. No such discrepancy was apparent when we used the indicator method at the time the readings with the gas-chain became constant. For the immediate determination of the H-ion

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<sup>1</sup> Levinson, A.: Arch. Pediat., 1916, 33, p. 241.

<sup>2</sup> Die Wasserstoffionenkonzentration, 1914.

<sup>3</sup> Jour. Am. Chem. Soc., 1913, 35, p. 847.



concentration we made use of the indicator method and for the old fluid we used either or both methods.

In preliminary experiments with the indicator method we followed the procedure recommended by Sörenson,<sup>4</sup> comparing the spinal fluid to the standard solutions described by Palmer and Henderson.<sup>5</sup> Since we had found that fluid examined immediately after withdrawal from the body was colorless to phenolphthalein, and since we could not use neutral red, the next indicator in the series, because of the difficulty of distinguishing color at night, the time when most of our punctures were performed, we made the phenolphthalein slightly alkaline by adding 1 c.c. of N/10 NaOH to 10 c.c. of phenolphthalein. We used 0.09 c.c. of the alkalinized phenolphthalein to 1 c.c. of spinal fluid, comparing this to 1 c.c. of the standard solutions. This showed a rough distinction between a H-ion concentration expressed in terms of  $P_H$  of 8.6, 8.0, 7.4, and 7.1.\* To distinguish the reaction of the spinal fluid still further we used the Levy-Rowntree-Marriott<sup>6</sup> standards, adding 0.2 c.c. of the indicator, (0.01% phenolsulphonephthalein) to 3 c.c. of spinal fluid, as recommended by the authors. On checking the standard color tubes against the gas-chain method both with spinal fluid and with borate and phosphate mixtures, we found that with our technic the readings with the colorimetric tubes corresponded to those of the gas-chain method up to a  $P_H$  of 8.1. After that point it was difficult to distinguish the quality of the color in the standard tubes, because of the difference in the intensity of the colors. Wherever possible, therefore, we used either the gas-chain method alone or both the gas-chain and the indicator method, on fluids standing for any length of time, placing more credence on the results with the gas-chain.

The spinal fluid we obtained was withdrawn by lumbar puncture in the usual manner, none but perfectly clear fluid being used by us in our work. The test tubes were closed with cotton plugs to conform with the usual clinical methods, except in the case of the special tests noted below. The fluids were kept at room temperature, usually about 20-23 C. The material in these investigations was secured from the Psychopathic Hospital and from the Michael Reese Hospital, both of Chicago. In this connection we wish to express our thanks to Dr. V. Finsand, resident physician of the Psychopathic Hospital, Dr. E. Armstrong of the County Hospital, and the staff of the Michael Reese Hospital for their cooperation in supplying us with material.

#### THE RESULTS WITH NONMENINGITIC FLUIDS

We examined 177 nonmeningitic spinal fluids drawn from 170 patients, including 20 cases of meningism, that is, cases that presented symptoms of meningitis, although the subsequent course of the disease proved them not to be meningitic in character. Of this number, 5 turned out to be cases of pneumonia, 2 of gastro-intestinal intoxication, 2 of nephritis, 2 of post-diphtheritic paralysis, 2 of poliomyelitis, 1 of tetanus, 1 of Little's disease, and 5 turned out to be meningism accompanying other infectious diseases. All of these occurred in children. Among the other cases there were 24 that proved to be cases of general paresis, 21 of alcoholic psychosis, 3 of dementia praecox,

\*  $P_H$ , as is well known, is a term recommended by Sörenson indicating the negative exponent of the H-ion concentration. Thus  $P_H 7.4 = N \times 10^{-7.4}$ . H-ions, being a fraction of a normal solution of free dissociated  $H^+$ . Therefore the higher the  $P_H$ , the lower the acidity.

<sup>4</sup> *Ergebnisse der Physiologie*, 1912, 12, p. 393.

<sup>5</sup> *Arch. Int. Med.*, 1913, 12, p. 153.

<sup>6</sup> *Arch. Int. Med.*, 1915, 16, p. 389.

TABLE 1  
H-ION CONCENTRATION OF NONMENINGITIC SPINAL FLUID

Case	Diagnosis	PH			
		Immediate	½ Hour	1 Hour	2 Hours
1	Little's disease.....	.....	.....	.....	.....
1	Little's disease.....	.....	.....	.....	.....
2	Tetanus.....	.....	.....	.....	.....
3	Pneumonia.....	.....	.....	.....	.....
4	Gastro-enteritis.....	.....	.....	.....	.....
5	Brain tumor.....	.....	.....	.....	.....
6	Endarteritis.....	.....	.....	.....	.....
7	Typhus.....	.....	.....	.....	.....
8	Brain tumor.....	.....	.....	.....	.....
9	Tic.....	.....	.....	.....	8.0 (b)
10	General paresis.....	.....	.....	.....	8.0 (b)
11	Delirium tremens.....	.....	.....	.....	.....
12	General paresis.....	.....	.....	.....	.....
13	Psychosis.....	.....	.....	.....	.....
14	Tubercle of brain.....	7.4 (b)	.....	.....	.....
15	Juvenile paresis.....	7.4 (b)	.....	.....	.....
16	Alcoholic.....	.....	7.4+(b)	.....	.....
17	General paresis.....	.....	7.4+(b)	.....	.....
18	Alcoholic.....	.....	.....	.....	.....
19	Paranoia.....	7.5 (c)	.....	.....	.....
20	Epilepsy.....	7.4 (c)	.....	.....	.....
21	Epilepsy.....	.....	7.5 (c)	.....	.....
22	C. Sp. Lues ?.....	.....	.....	7.9 (c)	.....
23	Paresis.....	.....	.....	7.7 (c)	.....
24	Psychosis.....	.....	.....	7.7 (c)	.....
25	Psychosis.....	7.5 (c)	.....	.....	.....
26	Psychosis.....	7.6 (c)	.....	.....	.....
27	Psychosis.....	7.5 (c)	.....	.....	.....
28	Psychosis.....	7.5 (c)	.....	.....	.....
29	Brain tumor.....	.....	.....	.....	8.0 (c)
					8.0 (b)
30	Pneumonia.....	.....	.....	.....	.....
31	Alcoholic.....	7.4 (c)	7.4 (c)	7.5 (c)	7.9 (c)
32	General paresis.....	7.4 (c)	.....	.....	.....
33	Psychosis.....	7.7 (c)	.....	.....	.....
34	Psychosis.....	7.5 (c)	.....	.....	.....
35	Alcoholic.....	7.4 (c)	.....	.....	.....
36	Psychosis.....	7.4 (c)	.....	.....	.....
37	Psychosis.....	7.5 (c)	.....	.....	.....
38	Alcoholic.....	7.5 (c)	.....	.....	.....
39	Alcoholic.....	7.4 (c)	.....	.....	.....
40	General paresis.....	7.5 (c)	.....	.....	.....
41	Psychosis.....	7.4 (c)	7.6 (c)	.....	.....
Mixture	.....	7.4 (c)	7.8 (c)	.....	.....
42	Psychosis.....	7.4 (c)	.....	.....	.....
43	Psychosis.....	7.5 (c)	.....	.....	.....
Mixture	Psychosis.....	7.4 (c)	.....	.....	.....
Mixture	.....	.....	.....	.....	.....
44	Poliomyelitis.....	.....	.....	7.7 (c)	.....
Mixture	General paresis.....	7.4 (c)	.....	.....	.....
45	.....	.....	.....	.....	.....
46	Pneumonia.....	7.7 (c)	.....	.....	.....
Mixture	.....	7.4 (c)	.....	.....	.....
47	.....	7.4 (c)	.....	.....	.....
48	Psychosis.....	7.5 (c)	.....	.....	.....
49	Meningism.....	7.5 (c)	.....	.....	.....
50	Poliomyelitis ?.....	.....	7.6 (c)	.....	.....

\* Corked.



2 of brain tumor, 1 of solitary tubercle of the brain, 1 of juvenile paresis, 2 of endoarteritis, 1 of epilepsy, and 1 of paranoia. The rest were cases of psychoses, the etiologic diagnosis of which was not established either clinically or by laboratory methods.

We found that the spinal fluids of nonmeningitic cases when examined immediately after withdrawal showed a greater acidity than that reported by other investigators. Our findings showed an H-ion concentration expressed in a  $P_H$  ranging from 7.4-7.6 on immediate examination. In only 2 cases of meningism did we get a  $P_H$  of 7.7. A positive Wassermann test, brain tumor, or even poliomyelitis seemed to have no effect on the H-ion concentration on immediate examination. The change in the H-ion concentration is so rapid, however, that in fluid examined 2 hours or later after removal from the body, we found the  $P_H$  to range from 7.9-8.1—the same as that reported by other investigators. We give in Table 1 cases showing our average results, indicating the method used by the letters a, b, c; "a" represents the gas-chain method, "b" the alkalinized phenolphthalein compared to the Sørensen standards, and "c" the Levy-Rowntree-Marriott<sup>7</sup> indicator compared to the standard colorimetric tubes. A typical change in the H-ion concentration is illustrated in Curve 1, together with the change in meningeal fluids. The results obtained by phenolphthalein compared to the Sørensen standards, are often marked with an additional + and — sign. This means that  $P_H$  of the spinal fluid was higher or lower than the figure given, but how high or how low was not determined.

The change on standing of nonmeningitic fluids is quite constant for the first 5 hours, reaching a  $P_H$  of 8.1. After that time, however, although the change is still fairly typical, it is not constant, being influenced by such factors as temperature and the amount of fluid in the tube and probably the content of  $CO_2$  in air. Some fluids rise to a  $P_H$  of 8.6, and sometimes 9, whereas others retain a  $P_H$  of 8.1. The effect of volume on the H-ion concentration we shall discuss later under the subject of mechanism.

Cavazzani<sup>7</sup> and Mott<sup>8</sup> draw attention to the fact that fluid drawn at night is more acid than that drawn during the day. The fluids we drew at night usually showed a  $P_H$  of 7.4-7.5, and the fluids drawn during the day generally showed a  $P_H$  of 7.5-7.6. Whether this difference is constant or not is a matter that requires further study.

#### DISCUSSION

Some of the earlier investigators who used the indicator method found the spinal fluid to be more acid than the commonly accepted  $P_H$  of 8.1. Cavazzani<sup>7</sup> found the spinal fluid of 2 cases of hydrocephalus to have a neutral reaction. Concetti<sup>9</sup> found the spinal fluid to be alkaline 3 times and weakly alkaline 4 times. Bisgaard<sup>10</sup> found the 2 cases of spinal fluid he examined immediately to be more acid than the borate mixture, 5.7 plus HCl, which is about 8.1. From this, Bisgaard concluded that phenolphthalein was not the proper indicator.

<sup>7</sup> Zentralbl. f. Physiol., 1896, p. 145.

<sup>8</sup> Lancet, 1910, 2, p. 1.

<sup>9</sup> Arch. f. Kinderheilk., 1898, 24, p. 161.

<sup>10</sup> Biochem. Ztschr., 1914, 58, p. 1.

Kopetszky<sup>11</sup> reports that all normal fluids are neutral to litmus paper. The H-ion concentration, according to his findings, must therefore be lower than  $P_{H}$  8. Especially significant is the work of John Turner<sup>12</sup> who not only knew the high H-ion concentration of the fluid, but was also aware of the fact that the concentration changes on standing. To quote him:

"In all the cases of this series and in 20 examined 15 years ago, I have obtained an alkaline reaction (and not amphoteric) to litmus paper, but with phenolphthalein the great majority have an acid reaction. The degree of acidity, however, is in many cases very slight. A very faint, pink solution of phenolphthalein was poured into two small beakers, so that the tint in both was similar in looking down at them as they stood upon a porcelain slab. A little of the fluid was then added to one beaker and generally the pink color was immediately discharged. I found that the fluid left unstoppered in my room, where gas is constantly burning, rapidly becomes alkaline, whereas similar fluid in stoppered bottles retained its acidity, and that in my later examinations where this source of fallacy was recognized and excluded, the results tend more and more to be uniformly acid with phenolphthalein."

On the other hand, there are a number of observers who employed more modern methods in the determination of the H-ion concentration and who found it to be low. Polyani<sup>13</sup> determined the H-ion concentration of one fluid drawn from a case of hydrocephalus, by the compensation method with the use of the Farkas-Szilisch electrode. He found the H-ion concentration to be  $9.084 \times 10^{11}$ . Hurvitz and Tranter,<sup>14</sup> who used the Levy-Rowntree-Marriott standards, found the  $P_{H}$  of spinal fluids to vary between 8.15 and 8.30 with an average of 8.26, this being somewhat lower when the fluid was dialyzed, the average then being 8.11. Weston<sup>15</sup> found the dialyzed fluid to vary between 7.9 and 8.3 with an average of 8.12.

We can account for the low concentration found by these investigators by the fact that their results were obtained on fluids that had been standing for some time. We, too, obtained a low H-ion concentration when we examined fluid that had been allowed to stand, and a high one when we examined fluids immediately on their withdrawal from the body. Very recently, in fact, during the preparation of this paper, Felton, Hussey, and Bayne-Jones<sup>16</sup> also made note of this fact. Their results, however, differ somewhat from our results, in that they

<sup>11</sup> Manhattan Eye, Ear and Throat Hospital Reports, 14, 1913.

<sup>12</sup> Jour. Ment. Sc., 1910, 56, p. 485.

<sup>13</sup> Biochem. Ztschr., 1911, 34, p. 205.

<sup>14</sup> Arch. Int. Med., 1916, 17, p. 828.

<sup>15</sup> Jour. Med. Research, 1916, 35, p. 367.

<sup>16</sup> Arch. Int. Med., 1917, 19, p. 1085.



found the  $P_H$  of fluids examined immediately to vary from 7.7-7.9, whereas we found the  $P_H$  to range from 7.4-7.6 on immediate determination.

#### RESULTS WITH MENINGITIC FLUIDS

In examining the fluids from cases of meningitis we employed the methods outlined for nonmeningitic fluids. We examined 74 specimens of 62 cases of tuberculous meningitis, 104 specimens of 62 cases of epidemic meningitis, and 12 specimens of 8 cases of pneumococcus meningitis. Among the cases of tuberculous meningitis we include in our records only those in which diagnosis was established either by the findings of tubercle bacilli in the spinal fluid or postmortem. All cases of epidemic and pneumococcus meningitis reported here were diagnosed by the finding of the respective bacteria in direct smear and culture.

We found that fluid from cases of tuberculous meningitis differed in no respect from that of normal cases, the  $P_H$  being 7.4-7.6 immediately after withdrawal and ascending to 8.1 or higher (Table 2). In some tuberculous fluids the H-ion concentration decreased in a much shorter time than in the normal fluid. Whether this rapidity of decrease in the H-ion concentration is generally characteristic of tuberculous fluids further experimentation will tell.

Fluid from cases of epidemic meningitis showed a H-ion concentration slightly higher than that of normal, when examined directly after withdrawal, the  $P_H$  being 7.2-7.5, the average being 7.3. The greatest deviation from normal and tuberculous fluid, however, was observed on fluid allowed to stand, the H-ion concentration then decreasing slowly in some cases, remaining stationary in others, and increasing in still others (Table 3). The more turbid the fluid, the longer it retained its acidity. The sicker the patient, the longer the fluid retains its acidity. Administration of serum alters the  $H^+$  concentration only in the cases that are improving (Table 4). The H-ion concentration of fluids of pneumococcus meningitis showed a strong resemblance to that of epidemic meningitis. However, owing to the small number of cases under our observation we shall not include them in our present series.

#### MECHANISM OF THE CHANGES OCCURRING IN SPINAL FLUID ON STANDING

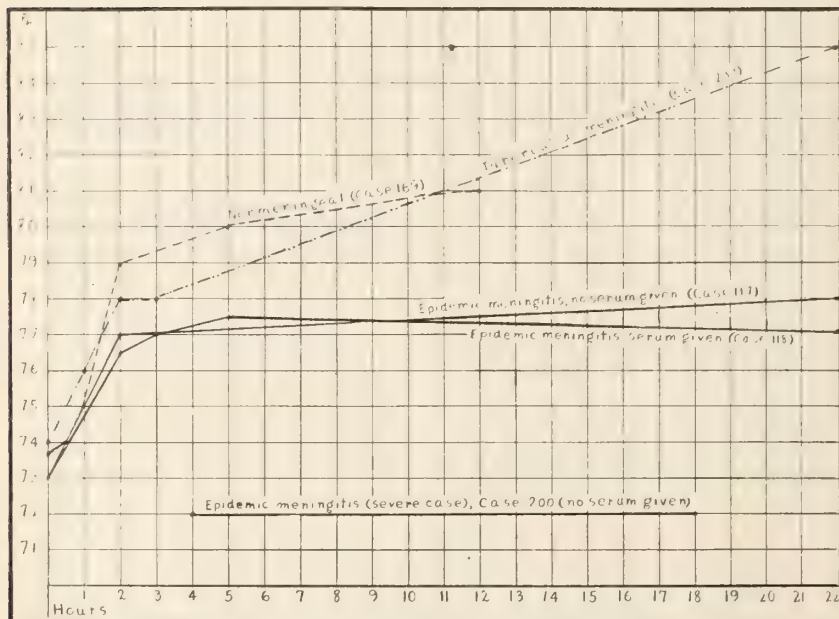
*Nonmeningitic.*—That changes in the acidity of spinal fluid occur on standing is not a novel finding. This phenomenon has been pointed out by Bisgaard,<sup>10</sup> Kopetzky,<sup>11</sup> Turner,<sup>12</sup> and Levinson.<sup>1</sup> In this work,



TABLE 4  
H-ION CONCENTRATION OF FLUID OF EPIDEMIC MENINGITIS AFTER ADMINISTRATION OF SERUM\*

Number	PH										
	Immediate	1/2 Hour	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	12 Hours	18 Hours	24 Hours	2 Days or Over
1	7.4-(b)										
2	7.4-(b)										
3	7.4-(b)										
4						7.0(b)					
5											7.4(b)
6									7.4(b)		
7								7.0(b)			
8		7.1(b)							7.4(b)		
9										7.1(c)	
10							7.4(c)				
11	7.3(c)										
12				7.7(c)							
13								7.6(c)	7.8(c)		
14											7.2(a)
15	7.1(c)								7.9(c)		
16									8.1*		
17	7.3(c)			7.6-7(c)		7.7-(c)	7.7-7.8(c)			7.7(c)	
18		7.4							8.2		

\* Diagnosis was not settled.



Curve 1. Change in H<sup>+</sup> concentration of 3 types of spinal fluids.

however, we were interested principally in finding the causes underlying the change. As spinal fluid is drawn into ordinary test tubes, we at first thought it possible that changes in acidity on standing might be due to the glass rather than the fluid. We discarded this idea, however, when we found that fluids left in nonsol glass also showed quantitatively the same degree of change. Having eliminated this possibility, we sought the cause of the change in the fluid itself.

There are only two possible sources for the changes taking place in the fluid on standing; one is the loss of volatile substances, such as  $\text{CO}_2$ , and the other is the formation of alkaline substances in the fluid, such as ammonia. To determine which is the factor responsible for the change, we instituted the following experiments:

We took a fluid immediately after its withdrawal from the body and divided it into 2 parts, 1 of which we examined directly and found its  $\text{P}_\text{H}$  to be 7.4, and the other portion we put in a beaker and introduced immediately into a desiccator containing 20%  $\text{NaOH}$ . We allowed the fluid to remain in the

TABLE 5

CHANGES IN  $\text{H}^+$  CONCENTRATION ON REMOVAL OF  $\text{CO}_2$  BY EXPOSURE TO ALKALI IN DESICCATOR

Case	Fluid Drawn	$\text{P}_\text{H}$ Immediately	Exposed to Alkali	Exposed to Air	Examined	$\text{P}_\text{H}$
190	6:55 p. m.	7.4	12 minutes		7:07 p. m.	7.7-7.8
190	6:55 p. m.	7.4	20 minutes		7:15 p. m.	7.8
190	6:55 p. m.	7.4	20 minutes	7 minutes after exposure to alkali	7:22 p. m.	7.8-7.9
190	6:55 p. m.	7.4	27 minutes	38 minutes after exposure to alkali	7:55 p. m.	7.8-9
190	6:55 p. m.	7.4	20 minutes	13 hours	11:22 a. m.	8.1
188	7:03 p. m.	7.4	10 minutes		7:13 p. m.	7.7-7.8
188	7:03 p. m.	7.4	30 minutes		7:33 p. m.	7.8
188	7:03 p. m.	7.4	30 minutes	3 minutes	7:36 p. m.	7.8
186	7:45 p. m.	7.4	6 minutes		7:51 p. m.	7.6-7.7
186	7:45 p. m.	7.4	25 minutes		8:10 p. m.	7.9

desiccator for periods varying from 10 to 30 minutes. After each removal from the desiccator we examined the fluid and found its acidity to be greatly decreased, from a height of 7.6-7.7 attained in 6 minutes to 7.8 in 20 minutes and 7.9 in 25 minutes, showing clearly that the removal of  $\text{CO}_2$  from the fluid has the effect of causing a decrease in the H-ion concentration in a short time (Table 5). We may state here that the fluid which had been left in the vacuum without  $\text{NaOH}$  also showed a rapid decrease in the H-ion concentration showing that the loss of  $\text{CO}_2$  thus accelerated under this condition decreased the H-ion concentration of the fluid in a similar way.

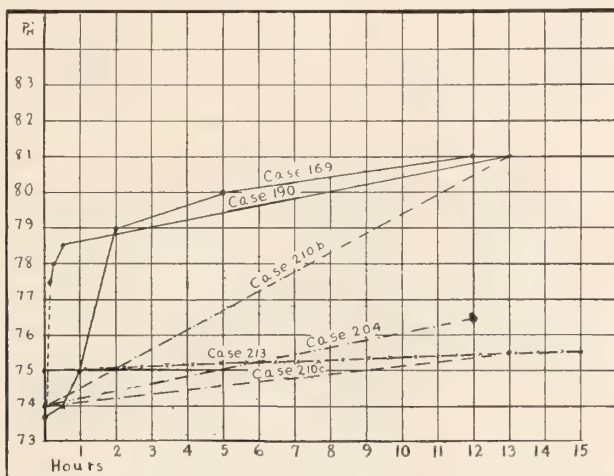
In order to ascertain whether the decrease in the H-ion concentration is due entirely to  $\text{CO}_2$  and no other factor, we corked the fluid tightly directly after its removal from the body. If the loss of  $\text{CO}_2$  is the sole cause of the decrease in the H-ion the tightly corked fluid ought to retain the original  $\text{P}_\text{H}$

**TABLE 6**  
**H<sup>+</sup> CONCENTRATION OF FLUIDS STANDING IN CORKED TUBES**

Number	Age	Diagnosis	Date Drawn	Date Examined	Interval	Stoppered With	pH
Mixture 210a	27	Alcoholic	p.m. 6/25/17 - 7:15	p.m. 6/25/17 - 7:15	Immediately		7.4
	38	Alcoholic	6/25/17 - 7:20	6/25/17 - 7:20	Immediately		7.4
210b	27	Alcoholic	6/ 5/17 - 7:15	a.m. 6/26/17 - 8:00	13 hours	Cotton	8.1
210c	27	Alcoholic	6/25/17 - 7:15	6/26/17 - 8:00	12 hours	Paraffined cork (few bubbles below cork)	7.5-7.6
	38	Alcoholic	6/25/17 - 7:20				
211a	43	Alcoholic	6/25/17 - 7:28	6/25/17	Immediately		7.5
	46	Alcoholic	6/25/17 - 7:37	6/25/17	Immediately		
211b	43	Alcoholic	6/25/17 - 7:28	6/26/17 - 8:10		Cotton	
	46	Alcoholic	6/25/17 - 7:37		12½ hours		8.1
211c	43	Alcoholic	6/25/17 - 7:28	6/26/17 - 8:10	12½ hours	Paraffined cork	7.5-7.6
	46	Alcoholic	6/25/17 - 7:37				
211d	43	Alcoholic	6/25/17 - 7:28	6/26/17 - 11:17	15½ hours	Paraffined cork replaced after 3 c.c. removed for exam. at 8:10, leaving 3 c.c. space in 6 c.c. tube	7.6
	46	Alcoholic	6/25/17 - 7:37				
212	55	Alcoholic	6/25/17 - 7:48	6/25/17 - 7:52	4 minutes		7.4
b	55	Alcoholic	6/25/17 - 7:48	6/26/17 - 11:05	15 hours	Paraffined cork	7.5-7.6
c	55	Alcoholic	6/25/17 - 7:48	6/26/17 - 11:30	15 hours	Cotton	8.2
d	55	Alcoholic	6/25/17 - 7:48	p.m. 6/28/17 - 12:30		Cotton	7.9
213	39	Alcoholic	6/25/17 - 8:00	6/25/17 - 8:02	2 minutes		7.5
b	39	Alcoholic	6/25/17 - 8:00	6/26/17 - 11:25	15½ hours	Paraffined cork	7.5-7.6
218	1 yr.	Pneumonia	6/26/17 - 1:30	6/26/17 - 1:30	Immediately		7.5-7.6
b	1 yr.	Pneumonia	6/26/17 - 1:30	6/28/17 - 12:30	47 hours	Paraffined cork	7.5-7.6
c	1 yr.	Pneumonia	6/26/17 - 1:30	6/28/17 - 12:30	47 hours	Cotton	8.2
204	46	Pulmonary tuberculosis	6/18/17 - 7:15	6/18/17 - 7:30	15 minutes		
	42	Alcoholic	6/18/17 - 7:20		10 minutes		7.4
	38	General paresis	6/18/17 - 7:30		Immediately		
b	38	General paresis	6/18/17 - 7:30	a.m. 6/19/17 - 7:30	12 hours	Cotton	8.1
c	38	General paresis	6/18/17 - 7:30	6/19/17 - 7:30	12 hours	Paraffin; left 3 c.c. space above volume of liquid	7.6-7.7
205	29	Alcoholic	6/18/17 - 7:40	p.m. 6/18/17 - 7:53	11 minutes		
	34	Alcoholic	6/18/17 - 7:45		8 minutes		7.4
	36	Alcoholic	6/18/17 - 7:53		Immediately		
b	36	Alcoholic	6/18/17 - 7:53	a.m. 6/19/17 - 7:55	12 hours	Cotton	8.1
c	36	Alcoholic	6/19/17 - 7:53	6/19/17 - 7:55	12 hours	Paraffined; 1 c.c. of space left on top of fluid	7.5-7.6



or be only slightly above it. If ammonia formation is responsible for the change the H-ion concentration ought to decrease. We found that fluid in tightly corked tubes with all the air excluded (no bubble on the top) retained its original  $P_H$ , proving that  $CO_2$  is the only factor responsible for the change (Table 6). We followed the experiment up further by filling the test tubes with different amounts of fluid, leaving various amounts of space between the fluid and the cork. In examining for the  $P_H$  we found that the greater the space above the fluid, the higher the  $P_H$  (Table 6). There is still a possibility that the increase in the  $P_H$  of fluid standing in a tube with cotton stopper may be due to the absorption of ammonia from the air. That this factor, if present, is negligible is shown by the following experiment: We divided a fluid into 2 portions, 1 of which we exposed to ammonia-free air in a desiccator, and the other we left in the ordinary laboratory air. After 30 minutes we examined the 2 fluids for their H-ion concentration and found it to be the same in both cases, showing that the usual decrease in the H-ion concentration of a fluid is not due to absorption of ammonia from the air (see Curve 2).



Curve 2. Change in  $H^+$  concentration of nonmeningitic fluids on standing at room temperature under various conditions. Case 169. Plugged with cotton. Case 190. Immediately after withdrawal, exposed to  $CO_2$  free air in desiccator (dotted line) and later left with cotton plug. Case 210b. Plugged with cotton; Case 210c, the same fluid as b, but corked with cork—a few bubbles at the top. Case 204. Six c.c. tube, half filled, and tightly corked; cotton plugged fluid was same as Case 210b (8.1). Case 213. Almost perfectly sealed with cork, without any air bubble above the fluid.

From these experiments it follows that if the spinal fluid is corked tightly the determination of the H-ion concentration can be made even hours after standing, provided no space is left for the escape of  $CO_2$ . When the tube is cotton-stoppered the amount of fluid in the tube and the portion taken must be ascertained.

*Tuberculous Meningitis.*—For the study of tuberculous fluids we tried similar experiments as those we had used for normal and we found, as we had in

the case of normal, that fluid in a tightly corked tube retained its original  $P_H$  (Table 7), indicating that the escape of  $CO_2$  is the principal factor determining the change on standing.

*Epidemic Meningitis.*—The slight but constantly higher H-ion concentration of the fluid in epidemic meningitis as compared to normal, on immediate examination, we believe, can be explained by the fermentation of the dextrose in

TABLE 7  
TUBERCULOUS FLUID, CORKED

Fluid	Date Drawn	Date Examined	Interval	How Stoppered	$P_H$
B.	6/28/17				7.4
B.	9:10	6/28/17 3:05	5 hours	Cork	7.4
B.		6/28/17 3:05	5 hours	Cotton	7.9
A. C.	7/13/17 11 a. m.	7/13/17 11 a. m.	Immediately		7.5-6 =
	7/13/17	7/14/17 11 a. m.	24 hours	Cork	7.5-6 =
	7/13/17	7/14/17 11 a. m.	24 hours	Cotton	8.0

TABLE 8  
FLUID FROM EPIDEMIC MENINGITIS, WITH CORK AND COTTON STOPPER

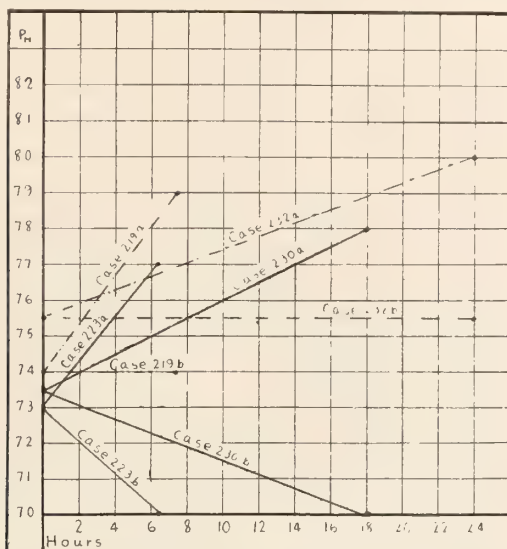
Diagnosis	Date Drawn	Date Examined	Interval	Stoppered With	$P_H$
Epidemic meningitis	6/30/17 12:20 p.m.	6/30/17 12:20 p.m. 6:35 p.m. 6:35 p.m.	Immediate  6¼ hours 6¼ hours	 Paraffined cork Cotton plug	7.3  7.0 7.7
Epidemic meningitis	7/ 6/17 2:30 p.m.	7/ 6/17 2:30 p.m. 7/ 7/17 8:30 a.m. 7/ 7/17 8:30 a.m.	Immediate  18 hours 18 hours	 Paraffined cork Cotton	7.4  7.3 8.2
Epidemic meningitis	7/11/17 11:30 a.m.	7/11/17  7/12/17 4:30 p.m. 4:30 p.m.	Immediate  17 hours 17 hours	 Paraffined cork Cotton	7.4-7.5  7.4 8.1
Epidemic meningitis	7/11/17 1:30 p.m.	7/11/17 1:30 p.m. 7/12/17 7:30 a.m.	Immediate  18 hours 18 hours	 Paraffined cork Cotton	7.3-7.4  7.0 7.8
Epidemic meningitis	7/14/17	7/14/17 7/15/17	Immediate 22 hours 22 hours	 Paraffined cork Cotton	7.3 7.1 7.9

the spinal fluid by the bacteria—a fact that has been brought out by several investigators. As for the mechanism underlying the slow decrease in the H-ion concentration of epidemic fluid, there are several possibilities to be considered, such as a slower loss of  $CO_2$  in the fluid on standing; a constant  $CO_2$  production by the cells present in the sediment of the fluid; lactic acid for-

mation due either to further fermentation of sugar by the bacteria in the test tube or to a destruction of cells on standing.

We found that the H-ion concentration in these fluids increased on standing in tightly corked tubes, from 7.4 to 7.0. This indicates that there is not only no loss of  $\text{CO}_2$ , but that there is also a formation of certain acids on standing, a fact quite different from nonmeningitic and tuberculous fluids (Table 8).

In order to study the cause of this increase of acidity the following experiments were made: We put 1 c.c. of tuberculous meningitic fluid in 1 chamber of the biometer (Tashiro<sup>17</sup>), and exactly the same amount of epidemic fluid in the other chamber of the biometer. We found that the tuberculous fluid gave off more  $\text{CO}_2$  than the epidemic, showing that although both tuberculous and epidemic fluids give off  $\text{CO}_2$  constantly, tuberculous fluid loses more  $\text{CO}_2$  than does epidemic. This would suggest that the increased acidity in epidemic meningitis is not due to a greater production of  $\text{CO}_2$ , but probably to the production of some other acid, very likely lactic acid (Curve 3).



Curve 3. Different effects of corking on tuberculous and epidemic meningeal fluids. Case 219a, tuberculous meningitis, cotton-plugged; b, same fluid, tightly corked (no air above). Case 232a, tuberculous meningitis, cotton-plugged; b, same fluid, corked (no air above). Case 223a, epidemic meningitis, cotton-plugged; b, same fluid, tightly corked (no air above); Case 230a, epidemic meningitis, cotton-plugged; b, same fluid, tightly corked (no air above).

#### SUMMARY

Spinal fluid of nonmeningitic cases covering a variety of conditions, is almost neutral, the  $P_H$  varying between 7.4-7.6 if the fluid is examined immediately on withdrawal from the body. The H-ion concentration of this fluid decreases steadily on standing. If the fluid is put into tightly corked tubes it does not change its H-ion concentration.

<sup>17</sup> A Chemical Sign of Life. 1917.

The decrease of acidity is most probably due to an escape of  $\text{CO}_2$  from the fluid.

Fluid from cases of tuberculous meningitis acts exactly like normal fluid, except that frequently the H-ion concentration shows a more rapid fall than the normal.

The H-ion concentration in epidemic meningitis varies according to the severity of the disease. Usually it is 7.3-7.4 immediately on withdrawal, decreasing very slowly. In some cases it even increases on standing with cotton plug. Put into a tightly corked tube, the fluid shows an increase in the H-ion concentration. The increase of the H-ion concentration of the fluid on standing is most likely due to a production of lactic acid in the fluid.

# ALKALOIDAL AND METALLIC PRECIPITATION OF CEREBROSPINAL FLUID IN THE DIAGNOSIS OF MENINGITIS

## STUDIES IN MENINGITIS. II

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The chemical tests that may be of diagnostic value in the differentiation of one form of meningitis from another are not numerous. The diagnosis as to the type of meningitis is based on bacteriologic findings generally. The bacteriologic tests when positive establish a definite diagnosis, but when negative they neither confirm nor rule out any disease, and it is left to make the diagnosis on the clinical and chemical data.

There are certain well-known diagnostic points that are of value in differentiating meningitis from nonmeningitic conditions, and one type of the disease from another.

In tuberculous meningitis, the spinal fluid is increased both in amount and pressure. The globulin reactions (Noguchi, Ross Jones, Nonne) are positive. The permanganate index is raised and the cells, which consist principally of lymphocytes, are increased in number. Most conclusive in this form of meningitis, is, of course, the demonstration of tubercle bacilli in the spinal fluid.

In epidemic meningitis, the fluid is almost always turbid and increased in amount and pressure, all globulin tests are positive, and the cells, which are mainly polymorphonuclear, are increased in number. The conclusive test in this form is the demonstration of meningococci in the fluid by means of smears and cultures. The same diagnostic means are employed in suppurative non-epidemic meningitis. Yet, in spite of all this, the diagnosis of the form of meningitis may remain unsettled. It is well-known that not only is it hard to find tubercle bacilli in smears of the fluid, but that often the globulin tests are negative until very late in the course of the disease. As to epidemic meningitis, sometimes the bacteria are few in number and sometimes they are not found at all. To grow the meningococci in cultures may be difficult, at times impossible. We therefore wish to present some results of chemical studies that may prove of corroborative value in differentiating meningitis from other conditions, and in separating the different forms from one another, principally tuberculous from epidemic meningitis.

Of the chemical and physico-chemical characteristics of spinal fluid, two stand out prominently in pathologic conditions, namely, protein content, and variation in the reaction of the fluid, or its hydrogen-ion concentration. As stated, globulin is increased in all meningeal inflammations. However, the amount of protein contained in various conditions gives us no criteria as to



the specific nature of the disease. Mestrezat<sup>1</sup> showed that in epidemic meningitis albumin may vary all the way from 0.15% to 0.85%, and in tuberculous meningitis from 0.12% to 0.56%. Thus it is evident that although epidemic meningitis usually has a higher protein content than tuberculous meningitis, the latter may at times have a higher protein content than the former. It is plain then that the protein content alone is not sufficient evidence on which to build the differentiation of the disease.

The other factor is the variation of the reaction of the fluid in different conditions, some fluids giving a high acidity and others a low one. Levinson<sup>2</sup> found that spinal fluid is alkaline to methyl red, that in tuberculous meningitis it gives practically the same index of alkalinity as normally, and that in epidemic and pneumococcus meningitis the fluid is much less alkaline than normally. Levinson's work, however, was done with fluid that had been standing for some time before being examined. Kopetzky<sup>3</sup> showed that normal fluid is neutral to litmus, but that in tuberculous and epidemic meningitis the fluid is acid to phenolphthalein, slightly less so in the tuberculous than in the epidemic disease.

TABLE 1  
SHOWING CATAPHORESIS OF PROTEINS; FIGURES TAKEN FROM MICHAELIS<sup>5</sup>

Substances	H <sup>+</sup>	Moves to
Casein.....	$4.9 \times 10^{-5}$ $4.1 \times 10^{-5}$ $1.3 \times 10^{-5}$	Cathode Stand still Anode
Serum Albumin.....	$1.2 \times 10^{-4} - 2.1$ $\times 10^{-5}$ $2.0 \times 10^{-5} - 1.9$ $\times 10^{-5}$ $1.1 \times 10^{-5} - 1.9$ $\times 10^{-5}$	Cathode Stand still Anode
Oxyhemoglobin.....	$2.4 \times 10^{-7}$ $1.2 \times 10^{-7}$	Cathode Anode
Gelatin.....	$1.5 \times 10^{-5}$ $1.2 \times 10^{-5} - 3.5$ $\times 10^{-5}$ $3.9 \times 10^{-5}$	Anode Stand still Cathode

As stated by Levinson (Page 556), we found that on immediate examination the H-ion concentration of fluid from tuberculous meningitis is exactly like that in nonmeningitic conditions and that H-ion concentration in epidemic meningitis is usually slightly higher than normal, remaining high for some time with a tendency to increase as the fluid stands. We also tried to show that the mechanism responsible for the high acidity in the fluid in epidemic meningitis after standing is the formation of some acid in the fluid, possibly lactic acid. One thing, however, seems certain, namely, the difference in the acidity of the fluid in tuberculous and epidemic meningitis. Sooner or later, a difference in the acidity of the two forms is apt to assert itself. It is this variation in acidity that we shall discuss now.

If the difference in the reaction of the different fluids is great enough, we should be able to distinguish their protein in a variety of ways, even if their

<sup>1</sup> Le Liquide Cephalo-Rachidien, 1912.

<sup>2</sup> Arch. Pediat., 1916, 33, p. 241.

<sup>3</sup> Manhattan Eye, Ear and Throat Hospital Reports, 1913, 14.

protein concentration might be the same. To make our point clearer, let us discuss briefly the precipitation reactions of the protein: For a more detailed discussion, see Mathews.<sup>4</sup>

It is well known that the electrical charge of a protein depends on the reaction of the medium. In acid solution, proteins become electropositive, and in alkaline solutions they become electronegative, as shown in Table 1. Thus, one of the ways of precipitating protein is to let it combine with some radicle to form an insoluble protein salt. From the standpoint of the electrical charge of the protein, such a protein salt is necessarily either a positive protein radicle forming a protein salt with negatively charged ions (alkaloidal precipitants), such as tungstic, picric, tannic acid and the like, or a negatively charged protein combining with a positively charged metal (metallic precipitants) such as Cu, Ag, Hg, Zn and Pb. If these protein salts are sufficiently insoluble, a precipitate will come down.

We thought it would be of interest to ascertain whether the difference in acidity of the spinal fluid under various conditions is great enough to produce different electrical states of the protein in the fluids. If it should be, it would be worth while to determine whether, by means of proper precipitants, we can distinguish one type of disease from the other.

#### EXPERIMENTS WITH CATAPHORESIS

Our first experiments were made to determine whether we could demonstrate electrically the existence of differently charged proteins in the spinal fluid of the two diseases. The apparatus we used was the one recommended by Michaelis<sup>5</sup> (Fig. 1). The fluids were placed in 3, care being taken to see that there was no bubble inside the core of the stopcocks. Tubes 2 and 4 were filled with 3% sulphosalicylic acid, and the upper portions (1 and 5), were filled with distilled water. To one electrode we added  $\text{CuCl}_2$  and to the other NaCl; we put a silver electrode in the anode and a copper electrode in the cathode. The poles were connected to a light circuit with 110 volt constant current, with an ordinary lamp in the circuit as resistance. The object was to determine the pole to which the protein would move. The migration of the protein from the spinal fluid was detected by the formation of a precipitate with sulphosalicylic acid in the arm. This method of course is necessarily crude. For an accurate test it would be necessary that the H-ion concentration, the osmotic pressure of the precipitants and of spinal fluid are the same for each experiment—a procedure almost impossible on account of the variation in the fluid. Furthermore, under these conditions, positively charged protein has a better chance to be precipitated at the cathode, while negatively charged protein, which moves to the anode, must change its charge at the anode in order to combine with sulphosalicylic acid. In other words, the amount of negatively charged protein to be precipitated at the anode depends not only on the amount of protein that moves to the anode, but also on the strength of the acidity of the precipitating agent at the anode.

However crude this method may be, it is convenient for detecting the charge of protein in spinal fluid. On this account, we used cataphoresis for testing the fluid of epidemic meningitis which shows a high acidity (Table 2).

As seen from the table, it is evident that in epidemic meningitis a considerable amount of protein moves toward the cathode, showing the presence of positively charged protein. In tuberculous meningitis we determined the

<sup>4</sup> Physiological Chemistry, 1916.

<sup>5</sup> Die Wasserstoffionenkonzentration, 1914.

charge of the protein of the fluid and found that in most cases it was at the anode, proving that there is more negatively charged protein in the fluid in this disease (Table 3). In nonmeningitic fluids there is very little precipitation in cataphoresis, only a slight diffusion being seen at one side or the other. Thus, it is possible to demonstrate that the existing variation in H-ion concentration of the spinal fluid is great enough to produce the different states of electrical charges of the protein. After this is established, it should be further possible to choose proper precipitants to distinguish the various forms of meningitis. In other words, if it is true that the variations are due to the differently charged proteins, we ought to be able to find more precipitate with a metallic precipitant than with an alkaloidal precipitant in tuberculous fluid, and in epidemic fluid the reverse would be the case.

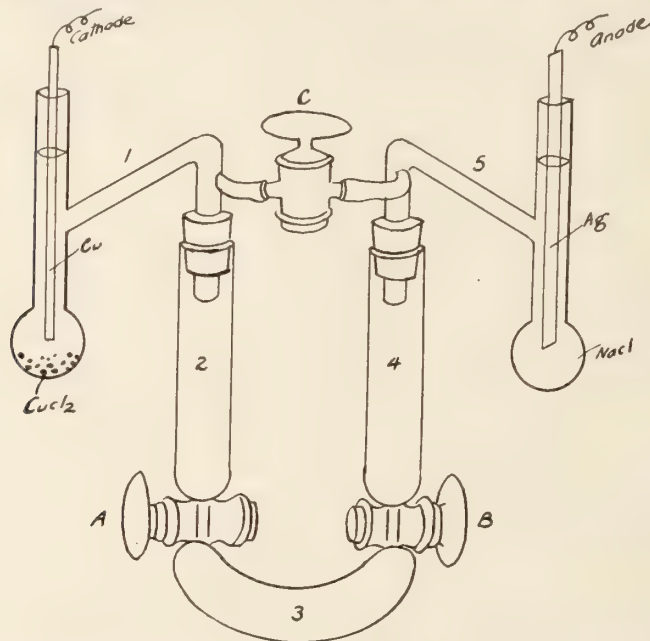


Fig. 1.—Apparatus for cataphoresis of proteids, after Michaelis, actual size.

The method we adopted was the following: We selected a 1% solution  $\text{HgCl}_2$  as the metallic precipitant, and a 3% sulphosalicylic acid as the alkaloidal precipitant.

We put 1 c.c. of a 3% sulphosalicylic acid in a small test tube about 7-8 mm. wide and 1 c.c. of a 1% mercuric chlorid in another tube of the same dimension. We added 1 c.c. of the same spinal fluid to each tube, believing that the more acid the fluid, the more precipitation there would be in the tube containing the sulphosalicylic acid, and the more alkaline the fluid the more precipitate there would be in the tube containing the mercuric chlorid.

We found that normal fluid gives only slight turbidity with sulphosalicylic acid and is clear with mercuric chlorid. The fluid on standing shows a little sediment at the bottom of the tube, the precipitate in the  $\text{HgCl}_2$  usually measuring 2.5-4 mm. in height and that in the sulphosalicylic acid 1-3 mm. (Table 4).

TABLE 2  
RESULTS OF CATAPHORESIS ON FLUIDS FROM EPIDEMIC MENINGITIS

Number	Interval	Cataphoresis		Remarks
		Cathode	Anode	
1 .....	1 day	↓	↓	
1 .....	1 day	↓	↓	
2 .....	20 hours	↓	↓	
2 .....	3 days	↓	↓	
3 .....	3 days	↓	↓	
3 .....	2 days	↓	↓	Fluid bloody
3 .....	3 hours	↓	↓	
4 .....	8 hours	↓	↓	Unusually alkaline fluid; cases brought in from another hospital; administration of serum not ascertained
5 .....	?	↓	↓	
6 .....	.....	↓	↓	
6 .....	17 hours	↓	↓	
7 .....	.....	↓	↓	
7 .....	2 days	↓	↓	

—> indicates heavy precipitate

↓ slight precipitate.

TABLE 3  
RESULTS OF CATAPHORESIS ON FLUID FROM CASES OF TUBERCULOUS MENINGITIS

Number	Time after Withdrawal	Cataphoresis	
		Cathode	Anode
1 .....	2 hours	↓	↓
1 .....	2 days	↓	↓
1 .....	2 days	↓	↓
2 .....	2 hours	↓	↓
2 .....	3 hours	↓	↓
3 .....	1 day	↓	↓
4 .....	3 hours	↓	↓
5 .....	24 hours	↓	↓

—> indicates heavy precipitate

↓ slight precipitate.

In tuberculous meningitis the sulphosalicylic acid gives a turbid precipitate immediately which settles to the bottom in several hours to a height of 3-6 cm. The mercuric chlorid is clear at first, but in 12-24 hours it settles in a gelatinous precipitate measuring 6-20 mm. (Table 5).

TABLE 4  
COMPARISON OF AMOUNT OF SEDIMENT FROM THE METALLIC AND ALKALOIDAL PRECIPITATION  
WITH NONMENINGITIC FLUIDS

Number	Diagnosis	Measurements of the Depth of Sediment in MM. after 24 Hours	
		HgCl <sub>2</sub>	Sulphosalicylic Acid
1	Psychosis	3	2
2	Tic.	4	3
3	General paresis	4½	2
4	Delirium tremens	3	1
5	General paresis	3½	2
6	Psychosis	3	2
7	General paresis	3½	2
8	General paresis	3	0
9	Psychosis	4	3
10	Psychosis	4	0
11	Psychosis	4	2
12	Meningism	3	2
13	Dementia præcox	3	0
14	Alcoholic	3	2
15	Psychosis	3	2
16	.....	4	2
17	Psychosis	3	1
18	Psychosis	2½	2
19	Meningism	3	2

TABLE 5  
AMOUNT OF SEDIMENT FROM THE METALLIC AND ALKALOIDAL PRECIPITATION FROM FLUID  
OF TUBERCULOUS MENINGITIS

Number	Measurements of the Depth of Sediment in MM. after 24 Hours	
	HgCl <sub>2</sub>	Sulphosalicylic Acid
1	10	4
1	15	4
1	10	3½
2	11	5
2	6	3
3	7	3
4	9	6
4	13	5
4	11	7
5	10	4
6	6	3
6	10	4
7	20	5

In epidemic meningitis before serum is given the sulphosalicylic acid produces a very marked turbidity, immediately forming a heavy precipitate. In several hours the precipitate settles to a height of from 7 to 20 mm. The mercuric chlorid, on the other hand, is clear immediately and shows a very slight



sediment on standing, ranging from 1 to 7 mm. in height, which in comparison with that produced by sulphosalicylic acid, is at a ratio of from 1:3, or even 1:5 (Table 6).

TABLE 6

MEASUREMENT OF SEDIMENT FROM METALLIC AND ALKALOIDAL PRECIPITATION FROM FLUID IN EPIDEMIC MENINGITIS BEFORE SERUM WAS GIVEN

Number	Measurements of the Depth of Sediment in MM. after 24 Hours	
	HgCl <sub>2</sub>	Sulphosalicylic Acid
1 .....	1	8
2 .....	2	7
3 .....	1	20
4 .....	1	10
5 .....	7	20

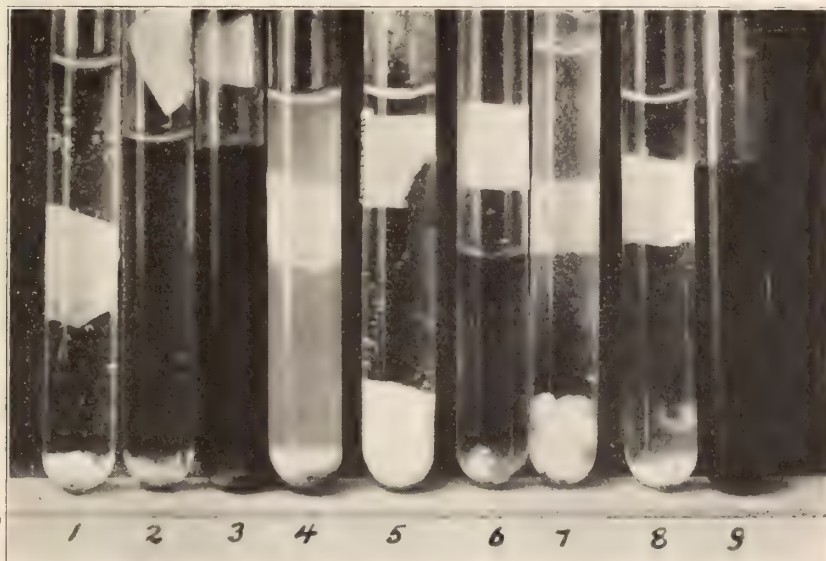


Fig. 2.—Photograph showing the typical ratio of precipitates by the 2 precipitants.

- 1.—1 c.c. of nonmeningitic fluid + 1 c.c. of 1 % HgCl<sub>2</sub>.
- 2.—1 c.c. of nonmeningitic fluid + 1 c.c. of 3 % sulphosalicylic acid.
- 3.—1 c.c. of nonmeningitic fluid + phenolphthalein.
- 4.—1 c.c. of fluid from epidemic meningitis + 1 c.c. of HgCl<sub>2</sub>.
- 5.—1 c.c. of fluid from epidemic meningitis + 1 c.c. of sulphosalicylic acid.
- 6.—1 c.c. of fluid from epidemic meningitis + phenolphthalein.
- 7.—1 c.c. of fluid from tuberculous meningitis + 1 c.c. of HgCl<sub>2</sub>.
- 8.—1 c.c. + 1 c.c. of sulphosalicylic acid.
- 9.—1 c.c. + phenolphthalein.

It should be noted that there is a slight excess of precipitate in the HgCl<sub>2</sub> tube over the sulphosalicylic tube in nonmeningeal fluids; that there is a much greater excess of HgCl<sub>2</sub> precipitate in tuberculous meningitis; and that in epidemic fluid the sulphosalicylic precipitate is in excess over the HgCl<sub>2</sub>.

After serum is given the difference between the two precipitants is not so marked as before but it is still marked enough to distinguish the fluid from the fluid of tuberculous meningitis, if the patient still shows marked meningeal symptoms.

It should be emphasized here that the principal feature in the precipitation experiment is not the amount of sediment in the tube, for this depends on the protein contents which vary considerably in different cases, but the important point is the ratio between precipitates obtained by mercuric chlorid and sulphosalicylic acid, a matter that depends chiefly on the H-ion concentration of the fluid.

It is interesting that the precipitation results are fairly constant regardless of the time that the fluid has been standing after withdrawal from the body. Since the H-ion concentration varies greatly on standing, but not very much on immediate examination, it may seem rather contradictory at first sight to have the precipitation not vary in different conditions. However, as we have tried to show, the gradual increase in the alkalinity of the fluid of tuberculous meningitis and of normal fluid is due to the loss of  $\text{CO}_2$ , while in epidemic meningitis there is an acid other than  $\text{CO}_2$  that is responsible for the gradual increase. It seems probable that when sulphosalicylic acid is added to fresh fluid in tuberculous meningitis, it hastens the escape of  $\text{CO}_2$ , a condition similar to the gradual loss of  $\text{CO}_2$  on standing; while in epidemic meningitis the loss of  $\text{CO}_2$  is not greatly affected by sulphosalicylic acid as the main acidity is probably due to a nonvolatile acid. For this reason the mercuric chlorid precipitate does not settle immediately as the H-ion concentration is high at the beginning. Thus, in tuberculous meningitis where the precipitate occurs it takes some time before it comes down.

Three typical results, representing nonmeningitic, tuberculous and epidemic meningitis, showing ratios of the precipitates obtained by two types of precipitating agents are shown in Figure 2.

#### SUMMARY

By a study of cataphoresis it is possible to show that there is a difference in the electrical charge of the protein in the fluids of the different forms of meningitis, the fluid in cases of epidemic meningitis containing more positively charged protein and the fluid in cases of tuberculous meningitis containing more negatively charged protein.

A convenient way of making a differential diagnosis of meningitis is to precipitate the spinal fluid by an alkaloidal, 3% sulphosalicylic acid, and a metallic, 1% mercuric chlorid, precipitant. All meningitic fluids show turbidity with the alkaloidal precipitant as soon as the test is made, thus indicating an inflammation of the meninges. On letting the precipitated fluid stand, a further differentiation is made by observing the ratio between the alkaloidal and metallic sediments. In normal fluid the sediment produced by either precipitant is slight, the sulphosalicylic acid sediment usually measuring 1-3 mm. in height and the mercuric chlorid 2-4 mm. in height. In tuberculous meningitis

the sediment of the mercuric chlorid precipitation is usually twice the height of the sulphosalicylic acid precipitation. In epidemic meningitis, on the other hand, the mercuric chlorid precipitation is slight and the sulphosalicylic acid precipitation is marked, the ratio of sulphosalicylic acid to mercuric chlorid ranging from 2:1 to 3:1. The ratio in epidemic meningitis is disturbed after serum is given.

# THE TOXIN OF BACILLUS WELCHII. I

## TOXIN PRODUCTION BY VARIOUS STRAINS

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Bull and Pritchett<sup>1</sup> were the first to demonstrate that *B. welchii* produces a soluble toxin. Metchnikoff,<sup>2</sup> Korentchevsky,<sup>3</sup> and others had obtained toxic substances, but these did not have the characteristics generally attributed to exotoxins. They were comparatively heat stabile and did not produce antitoxins on injection into the animal body. The first named investigators, by using a special medium and by incubating the cultures for a relatively short time were able to produce a toxin corresponding to those of diphtheria and tetanus, in that it was destroyed by heat of 60-70 C. and caused antitoxin production when injected into the animal body.

We have thought it desirable to test the toxin-producing ability of various strains of *Bacillus welchii*, and to determine the neutralizing power of one antitoxin against the toxins from these various strains. We have also looked into the question of toxin production by methods other than those used by Bull and Pritchett.

## THE COMPARATIVE TOXICOGENIC POWER OF TEN STRAINS OF BACILLUS WELCHII

The organisms used in these tests, with the exception of *B. perfringens*, and Strain 617d, were isolated in this laboratory. *B. perfringens* is the so-called "souche toxique" of Weinberg and Séguin, Strain 617d was received from Dr. Bull. This strain, which was the most powerfully toxicogenic of any of those used by Bull and Pritchett, was used to control the toxicity of the other strains.

Strains 1, 5, 7, 8, 9, 10, 11 and 17 were isolated from the stools of healthy individuals. A thick suspension of fecal material in 0.85% NaCl was made, filtration being resorted to for the removal of fecal masses; 0.5 c.c. of this suspension was placed in a tube of freshly boiled and cooled litmus milk. The mixture was at once heated to 80 C. for 60 minutes. The cultures were then incubated, and if the characteristic stormy fermentation developed, dilution tubes with 0.2% glucose agar were made according to the method of Veillon. The typical gas colonies growing in the depths of the medium were fished and transplanted into litmus milk.

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<sup>1</sup> Jour. Exper. Med., 1917, 26, p. 119.

<sup>2</sup> Ann. de l'Inst. Pasteur, 1908, 22, p. 929.

<sup>3</sup> Ibid., 1909, 23, p. 91.

Great confusion exists in regard to the criteria necessary to place an unknown organism in the species of *B. welchii*. It would be well then to sum up the more important characteristics of the strains just mentioned. All of the organisms are nonmotile, gram-positive rods with slightly rounded ends. They grow singly or in pairs, and when in the latter grouping exhibit a characteristic V formation. In the exudates of animals dying from infections, especially when these are produced by intraperitoneal inoculation, occasional long chains may be met with. The organisms all produce gas violently in dextrose agar and broth and give rise to the typical "stormy fermentation" in litmus milk. They do not form spores in carbohydrate medium, but do so readily in coagulated egg-white broth. The odor of butyric acid is very strong in milk and glucose-broth cultures. None of the cultures exhibit a putrefactive odor. Coagulated egg-white is not attacked even after several days at incubator temperature. The bacilli grown in milk and broth are very sensitive to the large amount of acid produced in such medium and survive for a few days at room or incubator temperature. Dead cultures may exhibit perfectly preserved forms, but the organisms, besides failing to grow on transplantation, will be found to have become gram-negative.

#### THE METHOD OF TOXIN PRODUCTION

The method used in the comparative tests was that of Bull and Pritchett<sup>1</sup> with a single modification. The medium was placed in 100 c.c. Erlenmeyer flasks, rather than in test tubes, as recommended by these authors. Fifty c.c. of 0.1% glucose broth were placed in each flask. Veal and Witte peptone were used in the preparation of the broth, and the reaction was adjusted to +0.5. Portions of sterile rabbit muscle, removed under aseptic conditions, were placed in the broth. The amount of muscle used was approximately equal to that of the liquid portion of the medium. Each flask was then inoculated with 1 c.c. of an 18-hour milk culture, covered with a layer of sterile paraffin oil, placed in a vacuum jar and exhausted to 28 inches. After such exhaustion the flasks were incubated at 37 C. for 18 hours. The medium was then removed under the oil by a bulb pipet and centrifuged to remove the bacilli and tissue debris. The supernatant fluid was filtered by suction through Berkefeld N filters and the filtrate was tested for sterility. The toxicity of the filtrates was then tested by intramuscular injection into guinea-pigs of 300 gm. weight.

All of the toxin tests recorded in Table 1 were made on the same day, from the same batch of medium. The toxin of Strain 617d was used as a control in these tests. It will be noted that the toxin produced by this organism was 3 times more powerful in Table 1 than in Table 2. In many different trials the potency of its toxin has varied between 0.05 and 0.5 c.c. The average variation, however, is not as



great as this. In the majority of cases, lethal doses of from 0.1 to 0.3 c.c. were obtained.

The abdominal surfaces of the guinea-pigs under test were carefully shaved. The injections were made into the muscles of the thigh. The standard for a lethal dose of the toxin was the smallest amount of toxin necessary to kill a 300 gm. guinea-pig in 48 hours. The standard used by Bull and Pritchett is 24 hours for a 350 gm. pigeon, but it has been found that while the guinea-pig does not react as rapidly as the pigeon there is no great difference in susceptibility to the toxin.

TABLE 1  
THE COMPARATIVE TOXIN PRODUCTION OF STRAINS 8, 9, 10 AND 617D

Strain	C.c. of Toxin	Result	Minimal Lethal Dose
8	1.0	+ 12 hours	0.5
	0.5	+ 19 hours	
	0.3	Severe necrosis	
	0.1	Moderate necrosis	
9	1.0	+ 10 hours	0.2
	0.5	+ 12 hours	
	0.3	+ 10 hours	
	0.2	+ 21 hours	
	0.1	Slight necrosis	
	0.05	0	
10	1.0	+ 12 hours	0.2
	0.5	+ 13 hours, 30 minutes	
	0.3	+ 21 hours, 30 minutes	
	0.2	+ 22 hours	
	0.1	Severe necrosis	
617d	1.0	+ 12 hours	0.1
	0.5	+ 12 hours	
	0.3	+ 10 hours	
	0.2	+ 11 hours	
	0.1	+ 21 hours, 30 minutes	
	0.05	Slight necrosis	

The findings at necropsy were similar to those recorded by Bull and Pritchett. The muscles at the site of inoculation are necrotic and there is a marked outpouring of gelatinous exudate over the subcutaneous abdominal surface. In animals that survive small doses of toxin extensive necrosis of not only the injected leg, but also the abdominal surface occurs. This is followed by sloughing and secondary infection.

Another series of tests was made with Strains 1, 5, 7, 11, 17, and B. perfringens. Graded doses were employed the same as in the experiments given in Table 1, but for the sake of brevity only the minimum lethal dose for each strain is given in Table 2.

It will be noted that in this case Strain 617d, which was used as a control, did not develop the most toxic filtrate. Strain 11 seems to be the most potent in this series. It is also worthy of note that the *B. perfringens* is a very poor toxin producer as compared with some of the strains isolated by us. Repeated tests of the toxicogenic power of this organism have been made, and in all cases the minimum lethal dose has varied between 1.0 and 2.0 c.c.

These results indicate that toxin production is a property common to many different strains of *B. welchii*. Not a single one of the 10 strains tested by us has failed to produce at least to some extent, a soluble toxic product. It is possible that subsequent work will show this to be a property so constant as to be of use in determination of the identity of the organism.

TABLE 2  
THE COMPARATIVE TOXICITY OF STRAINS 1, 5, 7, 11, 17, *B. PERFRINGENS*

Strain	Minimal Lethal Dose
1.....	0.5
5.....	0.75
7.....	1.0
11.....	0.1
17.....	2.0
<i>B. perfringens</i> .....	1.0
617d.....	0.3

It was of interest to ascertain whether all these toxins would be subject to neutralization by one and the same antitoxin. The antitoxin used in these experiments was kindly furnished us by Dr. Bull. One two-hundred and fiftieth of a c.c. protected a 350 gm. pigeon against 1 lethal dose. This amount, according to Bull, is designated as 1 unit of antitoxin. We did not determine whether the neutralizing power of this antitoxin was greater for guinea-pigs than for pigeons, but regarded the previously mentioned amount of antitoxin as 1 unit.

The tests given in Table 3 were made by mixing multiples of a guinea-pig lethal dose of toxin with corresponding units of antitoxin, allowing the mixtures to stand at the temperature of the room for 30 minutes, and at the end of this time injecting the mixture intramuscularly into guinea-pigs of 300 gm. weight. Controls of a similar number of lethal doses of toxin mixed with a corresponding amount of normal horse serum were made in every case.

The results given in Table 3 indicate that the antitoxin used neutralizes the toxins from every strain tested, and go far to show that the filtrates from the various strains are of a common nature. It will be noted that in many instances some signs of intoxication were present in the animals which had received toxin-antitoxin mixtures. These were in every case of a transient nature and were confined to slight or moderate edemas which disappeared and were not followed by any distinct necrosis.

TABLE 3  
NEUTRALIZATION OF VARIOUS TOXINS BY A SINGLE ANTITOXIN

Number Strain	C.c. of Toxin	Number of Minimal Lethal Doses	C.c. Antitoxin, 1:50	C.c. of Normal Horse Serum 1:50	Result
1	1.5	3	0.6	...	Nil
	1.5	3	...	0.6	+ 26 hours
5	1.5	2	0.4	...	Marked edema
	1.5	2	...	0.4	+ 15 hours
7	3.0	3	0.6	...	Nil
	3.0	3	...	0.6	+ 14 hours
8	2.5	5	1.0	...	Slight edema
	2.5	5	...	1.0	+ 16 hours
9	1.0	5	1.0	...	Very slight edema
	1.0	5	...	1.0	+ 17 hours
10	0.5	5	1.0	...	Slight edema
	0.5	5	...	1.0	+ 20 hours
11	0.3	3	0.6	...	Very slight edema
	0.3	3	...	0.6	25 hr., 30 min.
B. perfringens	2.0	2	0.4	...	Nil
	2.0	2	...	0.4	+ 16 hr., 30 min.
617d	0.5	5	1.0	...	Marked edema
	0.5	5	...	1.0	+ 15 hours

It would be desirable to test carefully the binding relations of this toxin and its antitoxin. While Bull and Pritchett have shown that this relation follows roughly the law of multiple proportions, it would seem extremely probable that the phenomenon of Bordet-Danysz would be encountered here, as in the case of tetanus and diphtheria toxins and their antitoxins. Plans for such determinations are at present under way.

#### VARIOUS METHODS OF PREPARING THE TOXIN

As antitoxin bids fair to have widespread use, especially during the war, consequently it would seem desirable to produce the toxin by as

simple a method as possible. We have accordingly studied toxin production under various conditions.

Tests were first made to determine the amount of sterile muscle necessary for the production of a potent toxin. Fifty c.c. of 0.1% glucose, +0.5 reaction, veal broth were placed in each of three Erlenmeyer flasks, A, B, and C. Sterile rabbit muscle was placed in Flask A in such amount as approximately to double the volume. Flask B received enough muscle to increase the volume by one fifth only. Flask C received no muscle. All 3 of the flasks were now inoculated with an 18-hour milk culture of Strain 617d, and after covering the surfaces with oil and exhausting in the usual manner, were incubated for 18 hours. Following this the cultures were filtered and centrifugated. The filtration was performed through a Berkefeld N filter. The injections were made intramuscularly. The results are given in Table 4.

TABLE 4  
THE RELATION OF QUANTITY OF STERILE MUSCLE TO TOXIN PRODUCTION

Set	Guinea-Pig		C.c. of Toxin	Result
	Number	Weight		
A	1	300	1.0	+ 8 hours
	2	300	0.5	+ 8 hours
	3	300	0.3	+26 hours
	4	300	0.1	Severe necrosis; recovered
B	5	300	1.0	+ 10 hours
	6	300	0.5	+ 10 hours
	7	300	0.3	+ 22 hours, 30 minutes
	8	300	0.1	Severe necrosis; recovered
C	9	300	2.0	+ 10 hours
	10	300	1.5	+ 22 hours
	11	300	1.0	Edema and moderate necrosis
	12	300	0.5	Slight necrosis

It will be observed from Table 4 that it makes no material difference whether large or small amounts of muscle are used. The lethal dose for A and B is the same. On the other hand, the toxicity of the culture without muscle was about a fifth as great as that of A and B.

We now attempted to determine the importance of paraffin oil in toxin production. It was found that very potent toxins can be obtained without the addition of this substance. In one case parallel tests were made of the toxicity of filtrates from 2 flasks, 1 of which had not been covered with oil. The toxicity of this filtrate reached the high value of 0.05 c.c. The filtrate from the oil-covered flask showed a lethal value of 0.1 c.c. This variation is of course too minute to be of impor-

tance, but the experiment indicated that the layer of oil was unnecessary in toxin production. Since this test was made we have never used the oil.

Other methods of anaerobiosis were tried with good results. Absorption of oxygen with pyrogallol proved to be efficacious, though no more so than the exhaustion method. Finally it was found that neither of these procedures was necessary, but that simple preliminary boiling of the medium before inoculation was all that was required for excellent growth of the organisms and resulting toxin production.

#### THE PRODUCTION OF HIGH TITER TOXIN WITHOUT STERILE MUSCLE

It was remarked previously that some toxin could be produced without the use of sterile muscle, the aseptic removal of which from

TABLE 5  
THE TOXICITY OF VEAL-MASH-BROTH FILTRATES (STRAIN 11)

	Number	C.c. of Toxin	Minimum Lethal Dose	Result
A Veal Mash-Broth (1:1)	1	1.0	...	+ 14 hours
	2	0.5	...	+ 15 hours
	3	0.3	...	+ 18 hours
	4	0.2	...	+ 40 hours
	5	0.1	0.1	+ 48 hours
B Veal Mash-Broth (1:2)	1	1.0	...	+ 12 hours
	2	0.5	...	+ 14 hours
	3	0.3	0.3	+ 21 hours
	4	0.2	...	Severe edema; recovered
	5	0.1	...	Slight edema; recovered
C Broth, No Veal Mash (Control)	1	1.5	...	+ 12 hours
	2	1.0	...	+ 12 hours
	3	0.5	...	+ 15 hours
	4	0.4	0.4	+ 36 hours
	5	0.3	...	Severe edema; recovered
	6	0.2	...	Severe edema; recovered
	7	0.1	...	Slight edema

rabbits introduces a factor of considerable complication into the method of toxin production, especially when this is to be conducted on an extensive scale. It would be highly desirable, however, to obtain a maximum yield of toxin and our attempts were directed toward this end.

Many workers experienced in the growing of anaerobic bacteria have found that suspensions of autoclaved minced beef or veal furnish an excellent medium for these organisms. At the suggestion of Dr. Novy we attempted the production of toxin by the substitution of autoclaved veal for the sterile rabbit muscle.



Strain 11 was used in this experiment, which was conducted as follows: A 0.1% glucose, + 0.5 reaction, veal broth was prepared. One hundred c.c. of this medium were placed in each of 3 flasks. To Flask A were added 100 gm. of finely chopped veal, to Flask B 50 gm., while no veal was added to the control Flask C. All 3 of the flasks were now autoclaved at 110 C. for 30 minutes. At once after cooling each flask was inoculated with 1.0 c.c. of an 18-hour milk culture of *B. welchii*, Strain 11. The cultures were then incubated, without exhaustion or covering with paraffin oil, for 18 hours. A heavy growth of characteristic organisms was observed in all of the flasks, although that in the flask without meat was not as rich as that in Flasks A and B. The fluid portion of the medium was now withdrawn by means of a bulb pipet, centrifugated at 8,000 revolutions per minute and filtered through Berkefeld N filters. The toxicity of the 3 filtrates was tested by intramuscular injection into guinea-pigs of 300 gm. weight. The result of the experiment is shown in Table 5.

It will be observed then, that this method furnishes an excellent substitute for that in which sterile rabbit muscle is used. It reduces considerably the complication of technic, and when a good toxin producing strain is used furnishes a toxin concentration as great as that obtained by the method of Bull and Pritchett (Table 2).

#### SUMMARY AND CONCLUSIONS

The toxicogenic power of 10 strains of *B. welchii* has been tested with the result that all the strains have been found to produce toxin in greater or less degree.

An antitoxin produced by the injection of toxin from a single strain is capable of neutralizing all of the toxins. This fact furnishes strong evidence of the common nature of the toxic products of the various strains.

Various methods of toxin production have been tested. It has been determined that a layer of sterile paraffin oil is not necessary for securing growth or toxicity. Methods of removing oxygen, such as exhaustion or absorption are superfluous. It is necessary only to boil the medium before use.

A considerable concentration of toxin may be secured without the addition of sterile muscle. The lowest minimum lethal dose secured by this method has been up to the present, 0.4 c.c. The method of choice which combines simplicity with a comparatively high concentration of toxin, is the veal mash-broth cultivation we described. In this method the complicating factor of aseptic muscle is avoided.

# THE TOXIN OF *BACILLUS WELCHII*. II

## THE MECHANISM OF INFECTION WITH *B. WELCHII*

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It is generally accepted that the human body is highly resistant to infection by *Bacillus welchii*. Westenhoeffer,<sup>1</sup> for instance, claims that this organism is a pure saprophyte and is able to multiply in dead tissue only. It is the experience of surgeons that gas gangrene infections establish themselves only after severe trauma, which damage tissues badly and cut off the circulation from certain areas. This explains the first stage of the infection, but leaves its subsequent extension unaccounted for. It is possible that the analysis of the conditions of experimental infection in animals might throw light on this question.

Guinea-pigs are susceptible to infection with the majority of strains of *B. welchii*, but it is well known that comparatively large amounts of the culture have to be injected to produce infection. The opinion is generally held that this is due to the injection of the highly acid culture medium along with the organisms. The acid is present in a concentration sufficient to damage the tissue, thus preparing it for the growth of the organism. Hitschmann and Lindenthal<sup>2</sup> claim that the virulence of different strains of *B. welchii* varies with their ability to produce acid. Simonds<sup>3</sup> was unable to produce infection in rabbits when he used large masses of bacilli taken from the surface of agar bottle slant cultures. He ascribes this failure to the fact that no acid or metabolic products, as would have been the case with liquid cultures, were injected along with the organisms.

The discovery by Bull and Pritchett<sup>4</sup> of the production of a soluble toxin by *B. welchii* suggests another explanation of the mechanism of infection. It is possible that the tissue damage which paves the way for the experimental infection and results in its rapid extension may not be due to the acid at all, but rather to the injection, along with the organ-

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<sup>1</sup> Virchow's Archiv., 1902, 170, p. 517, quoted from Simond.<sup>3</sup>

<sup>2</sup> Sitzungsber. d. k. Akad. d. Wissensch., math.-naturwissensch. Kl., 3te Abt., 1899, 108, p. 67, quoted from Simonds.

<sup>3</sup> Studies in *Bacillus welchii*, etc., Monographs of the Rockefeller Institute for Medical Research, No. 5, 1915.

<sup>4</sup> Jour. Exper. Med., 1917, 26, p. 119.

ism, of small amounts of toxin. This toxin has a strong necrotic effect on muscular tissue. It is likely then, that it may play the rôle of an aggressin, not in the sense of Bail, but rather as an agent which establishes conditions suitable for the growth of the "necro-parasitic" bacillus. This property of the toxin might account not only for the establishment of experimental infections but for the extension of infections in the human body. It is our purpose to show that this is indeed the case and that acid plays a small if any part in the experimental infection.

#### THE VIRULENCE OF NEUTRALIZED CULTURES

The organism used in this test was *B. welchii*, Strain 617d. The culture medium was a 0.2% glucose veal broth, incubated 18 hours. Three tubes of the 18-hour culture which showed a heavy growth of typical organisms were pooled, and subsequently divided into 2 parts. Part "A" was neutralized with normal

TABLE 1  
THE VIRULENCE OF NEUTRALIZED CULTURES

Exper.	Guinea-Pig		C.c. of Neutral Culture	C.c. of Acid Culture	Result
	Number	Weight			
A	1	300	1.5	...	+ 14 hours; gas, edema
	2	300	1.0	...	+ 14 hours; gas, edema
	3	300	0.5	...	+ 17 hours; gas, edema
	4	300	0.2	...	+ 16 hours; gas, edema
	5	300	0.1	...	+ 17 hours; gas, edema
	6	300	0.01	...	Nil
	7	300	0.005	...	Nil
B	1	300	...	0.5	+ 17 hours; gas, edema
	2	300	...	0.1	+ 24 hours; gas, edema
	3	300	...	0.01	Nil
	4	300	...	0.005	Nil

NaOH; 0.08 c.c. of the normal soda per c.c. was required to neutralize the culture to phenolphthalein. Part "B" remained unneutralized and served as the control. The hind-legs and the abdominal surfaces of the guinea-pigs used were shaved, this procedure facilitating the observation of the course of infection. The injections were made with a fine needle deep into the thigh muscles, and were carried out at once after the neutralization of Part "A."

The first evidence of infection is the swelling of the injected leg, which is invariably painful to palpation. The swelling, which is edematous, rapidly spreads into the groin and finally into the abdominal surface.

Palpation of the leg and inguinal region reveals crepitation. Although we have observed recovery of guinea-pigs after infections in which the edema had extended to the abdominal surface, the presence of gas invariably indicates a fatal infection. The rapidity of death depends to a great extent on the amount of culture material injected. It varies with this factor from 4-48 hours. The result of this experiment is recorded in Table 1.

This experiment indicates that the virulence of neutralized cultures differs little if any from those not neutralized. In the case of both

"A" and "B" 0.1 c.c. of culture resulted in fatal infection while 0.01 c.c. failed to do so." It was not thought necessary to introduce values between 0.1 and 0.01 c.c., as such differences would be negligible when slight variations in individual animals are taken into account. If the culture medium possesses some injurious property which allows the organism to gain a foothold, it must be ascribed to some other factor than the acidity.

#### INFECTION WITH SUPERNATANT FLUIDS OF CENTRIFUGATED CULTURES

In studying the effect of the medium in which *B. welchii* has grown on the virulence of the organism it would be desirable to separate the medium from the organisms. In all subsequent experiments the organisms used for infection were separated from the culture medium by centrifugation and the resulting bacillary sediment was washed and

TABLE 2  
INFECTIVITY OF CULTURE SUPERNATANT FLUIDS AFTER CENTRIFUGATION AT 8,000 R. P. M.

Super- natant	Guinea-Pig		C.e. Inject- ed Intra- muscularly	Result
	Number	Weight		
I	1	250	1.0	+ 5 hours, 30 minutes; gas, edema*
	2	250	5.0	+ 3 hours, 45 minutes; gas, edema*
II	1	700	1.5	+ 22 hours; gas, edema*
	2	700	2.0	+ 21 hours; gas, edema*

\* Necropsy typical. Numerous bacilli in exudate.

centrifuged twice with large volumes of sterile 0.85% NaCl solution. The centrifugation was in all instances carried out at 8,000 revolutions per minute. The organism sediment from the 2nd washing was suspended in an amount of NaCl solution equal to the original amount of the culture. The resulting suspension represented, c.c. for c.c., the number of organisms in the original culture. The sediments during the washing and when suspended before injection were carefully agitated in a bulb pipet to avoid the presence of clumps of organisms. The NaCl solution used for washing and final suspension was kept in ice in order to preserve as far as possible the viability of the organisms.

Simonds<sup>3</sup> states that 5 c.c. of a supernatant fluid from an egg-broth culture still contained, after 30 minutes of centrifugation, enough organisms to kill a guinea-pig in 21 hours. Bull and Pritchett<sup>4</sup> also note the fact that a few organisms left behind in the supernatant fluid after centrifugation were sufficient to cause infection. It would seem

necessary, therefore, to test the infectivity of supernatant fluids from cultures even after the violent centrifugation described above. If this were not done, the supposed effect of culture supernatants on washed organisms might be in reality due to the addition of organisms which failed to sediment.

This question was attacked in the following experiment. The supernatant fluid from an 18-hour 0.2% glucose broth culture of Strain 617d was neutralized to phenolphthalein with normal NaOH. The fluid had been obtained from the broth culture by 10 minutes of centrifugation at 8,000 r. p. m. The injections were made intramuscularly. Table 2 represents the results of 2 different experiments made with different supernatant fluids.

This experiment confirms the observations of the workers referred to. It indicates, moreover, that it would be unsafe to use a merely centrifugated culture supernatant. It is necessary to remove completely all organisms from the fluid before attempting to analyze the comparative importance of these 2 factors in infection. In all subsequent experiments the cultures were neutralized, centrifugated and filtered through Berkefeld N filters. The filtrates were then tested for sterility before use.

#### THE EFFECT OF CULTURE FILTRATES ON THE VIRULENCE OF WASHED B. WELCHII

Having determined the unimportance of acid as a factor in the initiation of infection, it was thought desirable to determine in a roughly quantitative manner the aggressive effect of the neutralized filtrates.

Three tubes of an 18-hour 0.2% glucose broth culture of Strain 617d were pooled, neutralized, centrifugated and filtered. The filtrate proved to be free from organisms. The sediment of bacilli was washed twice with large volumes of 0.85% NaCl solution and after the 2nd washing was suspended in a volume of NaCl solution equivalent to the original culture.

The suspension was now diluted 1:10, 100, 1,000, 10,000, and 100,000 in cold sterile NaCl solution. Each dilution was shaken carefully to insure perfect suspension. In the case of tests 7, 8, and 9, in both experiments A and B, 0.2, 0.3 and 0.5 c.c. of the original suspension were brought to 1 c.c. volume with sterile NaCl solution. In the rest of the tests 1 c.c. of the dilutions mentioned was injected.

*Experiment A.*—One c.c. of each dilution was mixed with 0.5 c.c. of neutral culture filtrate, drawn up into a syringe and injected intramuscularly.

*Experiment B (Control).*—One c.c. of each dilution was mixed with 0.5 c.c. of sterile 0.2% glucose broth and injected as in A.

*Experiment C.*—The culture filtrate was tested for toxicity.

The injections in the case of Expts. A and B were made in order from the higher to the lower dilutions. The guinea-pigs used weighed 250 gm.



Observation of Table 3 shows that the neutral filtrate is able to increase the virulence of the washed bacillus at least 10,000 times. It is unfortunate that higher dilutions in Exper. A were not made and

TABLE 3  
THE EFFECT OF CULTURE FILTRATE ON WASHED ORGANISMS (617D)

Exper.	Number	C.c. of Washed Organisms	C.c. of Filtrate	C.c. of Broth	Result
A	1	0.00001	0.5	...	+ 19 hours; edema, gas
	2	0.0001	0.5	...	+ 11 hours; edema, gas
	3	0.001	0.5	...	+ 18 hours; edema, gas
	4	0.01	0.5	...	+ 15 hours; edema, gas
	5	0.05	0.5	...	+ 16 hours; edema, gas
	6	0.1	0.5	...	+ 14 hours; edema, gas
	7	0.2	0.5	...	+ 14 hours; edema, gas
	8	0.3	0.5	...	+ 13 hours; edema, gas
	9	0.5	0.5	...	+ 14 hours; edema, gas*
B	1	0.00001	...	0.5	0
	2	0.0001	...	0.5	0
	3	0.001	...	0.5	0
	4	0.01	...	0.5	0
	5	0.05	...	0.5	0
	6	0.1	...	0.5	+ 36 hours; edema, gas*
	7	0.2	...	0.5	+ 16 hours; edema, gas
	8	0.3	...	0.5	+ 16 hours; edema, gas
	9	0.5	...	0.5	+ 15 hours; edema, gas
C	1	...	0.5	...	0
	2	...	0.75	...	0
	3	...	1.0	...	0

\* Necropsies typical.

tested, since it is possible that still fewer organisms, under the influence of the filtrate might have proven fatal. On the other hand, it is certain that the neutral filtrate possess enormous aggressive power. This experiment was repeated with equally good results.

It seemed desirable to test the effect of the filtrate on another strain of *B. welchii*. Strain 7, isolated in this laboratory from the stool of a normal individual was used in this experiment.

TABLE 4  
THE EFFECT OF CULTURE FILTRATE ON WASHED ORGANISMS, STRAIN 7

Exper.	Number	C.c. of Washed Organisms	C.c. of Filtrate	C.c. of Broth	Result
A	1	0.0001	0.5	...	+ 19 hours; gas, edema
	2	0.001	0.5	...	+ 17 hours; gas, edema
	3	0.01	0.5	...	+ 16 hours; gas, edema
	4	0.1	0.5	...	+ 15 hours, 30 minutes; gas, edema
	5	1.0	0.5	...	+ 14 hours; gas, edema
B	1	0.05	...	0.5	0
	2	0.1	...	0.5	0
	3	0.2	...	0.5	0
	4	0.3	...	0.5	0
	5	0.5	...	0.5	+ 18 hours; gas, edema
	6	1.0	...	0.5	+ 21 hours; gas, edema

Injections were made intramuscularly. The guinea-pigs weighed 250 gm.

An 18-hour culture of this strain in 0.2% glucose broth was centrifugated and the suspension made in the usual manner. The organisms were washed twice and the final suspension was made up to original volume with 0.85% NaCl solution. The mixing of the filtrate and the broth (control) with the dilutions of the washed organisms was carried out in a manner exactly similar to that of the preceding experiment.

The aggressive action displayed by the filtrate in regard to Strain 617d is seen also to be present in the case of Strain 7. In the latter case the virulence of the organism is increased by at least 5,000 times. One ten thousandth of a c.c. of organism suspension kills when mixed with the neutral filtrate, while 0.5 c.c. is required to infect when mixed with plain glucose broth.

In the preceding experiments constant amounts of culture filtrates were used together with varying amounts of organisms. The next inquiry concerned the amount of the aggressive filtrate required to make a given amount of the washed organism suspension virulent. The amount of washed organisms chosen was 0.01 c.c., about one-tenth the amount usually required to infect when no aggressin is used.

TABLE 5  
EFFECT OF VARYING AMOUNTS OF AGGRESSIVE FILTRATE ON THE VIRULENCE OF B.  
WELCHII (617D)

Exper.	Number	C.c. of Washed Organisms	C.c. of Filtrate	C.c. of Broth	Result
A	1	0.01	0.5	...	+ 18 hours; edema, gas
	2	0.01	0.3	...	+ 17 hours; edema, gas
	3	0.01	0.1	...	Mild infection, recovers
	4	0.01	0.075	...	0
	5	0.01	0.05	...	0
B	1	0.01	...	0.5	0
	2	0.01	...	0.3	0
	3	0.01	...	0.1	0
C	1	....	2.0	...	Severe edema, recovers
	2	....	1.5	...	Moderate edema
	3	....	1.0	...	0

The organism used was Strain 617d washed twice and suspended in sterile NaCl solution as usual. One c.c. of a 1:100 dilution of the organism suspension was mixed with varying amounts of the neutral filtrate. As controls, similar amounts of organism plus similarly varying amounts of 0.2% glucose broth were used. Controls of filtrate toxicity were also made.

The guinea-pigs used in this experiment weighed about 200 gm. The injections were made, as usual, intramuscularly. The result of the experiment is given in Table 5.

Reference to Experiment A in Table 5 shows that 0.3 c.c. is the smallest amount of supernatant fluid that will cause 0.01 c.c. to set up a fatal infection. Observation of Experiment C will indicate that the dose of filtrate having an aggressive action is far below the fatal toxic

dose. This does not mean, however, that the toxin and the aggressin are not one and the same thing. As we have remarked before, non-fatal doses of toxin will give rise to severe local necrotic effects.

#### THE SPECIFIC NATURE OF THE AGGRESSIN

We have shown that considerable concentrations of toxin can be obtained from 0.2% glucose broth without the addition of sterile muscle. It is entirely possible then that the aggressin may be identical with the toxin. The following experiment appears to show that this is in all probability the case.

TABLE 6

THE EFFECT OF HEAT AND OF ANTITOXIN ON THE AGGRESSIVE POWER OF NEUTRAL FILTRATES

Exper.	Number	C.c. of Washed Organisms	C.c. of 70 C. Filtrate	C.c. of Unheated Filtrate	C.c. of 1:50 Anti-toxin	C.c. 1:50 Horse Serum	Result
A	1	0.01	0.5	...	...	...	0
	2	0.001	0.5	...	...	...	0
B	1	0.01	...	0.5	...	...	+ 19 hours*
	2	0.001	...	0.5	...	...	+ 20 hours*
C	1	0.01	...	0.5	1.0	...	0
	2	0.001	...	0.5	1.0	...	0
D	1	0.01	...	0.5	...	1.0	+ 20 hours*
	2	0.001	...	0.5	...	1.0	+ 32 hours*

\* Typical necropsy; gas, copious edema, many bacilli.

The organisms used were washed bacilli from an 18-hour glucose broth culture of Strain 617d. The neutral filtrate was secured from the supernatant fluid of this same culture.

*Experiment A.*—A small amount of the neutral filtrate was heated to 70 C. for 30 minutes and 0.5 c.c. were added to each of 2 tubes, containing 1 c.c. of 1:100 and 1:1,000 respectively of washed organisms. The mixtures were shaken thoroughly and injected at once intramuscularly.

*Experiment B (Control).*—There were added 0.5 c.c. of unheated filtrate to each of 2 tubes, containing the same amounts of bacilli as in Exper. A, mixed, and injected at once.

*Experiment C.*—In each of 2 tubes 0.5 c.c. of unheated filtrate was placed and to each of these were added 5 units of Welch antitoxin (1 c.c. of 1:50). The tubes were allowed to stand at room temperature for 30 minutes, then 1 c.c. of 1:100 and 1:1,000 washed organisms respectively were added to Tubes 1 and 2. The mixtures were shaken thoroughly and injected intramuscularly.

*Experiment D (Control).*—In Tubes 1 and 2, 0.5 c.c. of unheated filtrate was placed. Then 1 c.c. of 1:50 normal horse serum was added to each, and the mixtures allowed to stand for 30 minutes. To Tubes 1 and 2 were added 1 c.c. of 1:100 and 1:1,000 respectively of washed organisms. The mixtures were shaken and injected at once intramuscularly.

The guinea-pigs used in this experiment weighed 250 gm. The result is given in Table 6.

The result of this experiment is illuminating. Whereas in Experiment A Nos. 1 and 2, which received organisms treated with 70 C. filtrate, showed no sign of infection, the controls in Experiment B, Nos. 1 and 2, died promptly with edema and gas. Necropsy showed myriads of Welch bacilli at the site of inoculation and in the exudate on the abdominal surface. In the case of Experiment C, Nos. 1 and 2, in which the filtrate was treated with 5 units of antitoxin, no infection took place, while the controls in Experiment D, Nos. 1 and 2, died with typical infection just as did the controls, Nos. 1 and 2 of Experiment B.

The aggressin, therefore, is destroyed by heat of 70 C., and is neutralized by antitoxin, and it may be safely concluded that true toxin, present in small amounts in the neutral filtrates, is responsible for the aggressive action. It would be of interest to determine whether the toxin made in the usual manner and capable of killing in small doses, would exert a similar effect when injected together with washed organisms.

TABLE 7  
THE AGGRESSIVE ACTION OF TOXIN FROM MUSCLE CULTURES

Exper.	Number	C.c. of Washed Organisms	C.c. of Filtrate	C.c. of Anti-toxin 1:250	C.c. of Normal Horse Serum 1:250	Result
A	1	0.01	0.1	1.0	...	0
	2	0.01	0.05	1.0	...	0
	3	0.01	0.01	1.0	...	0
B	1	0.01	0.1	...	1.0	+ 48 hours; gas, edema*
	2	0.01	0.05	...	1.0	Severe infection, recovered
	3	0.01	0.01	...	1.0	0

\* Necropsy typical, many bacilli.

The toxin employed in this experiment was made as follows: 0.1% glucose veal broth was placed in a small Erlenmeyer flask and several pieces of freshly excised sterile rabbit muscle were added. The flask was then inoculated with about 1 c.c. of an 18-hour litmus milk culture of Strain 617d, exhausted, and after 18 hours' incubation filtered through a Berkefeld N. filter. The minimum lethal dose for guinea-pigs of 300 gm. weight was 0.3 c.c.

*Experiment A.*—In each of 3 tubes 0.1, 0.05, and 0.01 c.c. of the above toxin were placed and mixed with 1 c.c. of a 1:250 Welch antitoxin (1 unit). The mixtures stood at the temperature of the room for 30 minutes; then to each tube was added 0.01 c.c. of a washed suspension of 617d strain. The mixtures, after shaking, were immediately injected intramuscularly.

*Experiment B (Control).*—To 3 tubes containing the same amounts of toxin as in Experiment A, 1 c.c. of 1:250 normal horse serum was added. After 30 minutes, 0.01 c.c. of the washed organisms was added to each, and the mixture injected as in Experiment A. The guinea-pigs used in this experiment weighed 300 gm. The result of the experiment is given in Table 7.

As in the preceding experiment the toxin mixed with antitoxin has no aggressive power while similar amounts which have been treated with normal horse serum cause in the case of 0.1 c.c. fatal and of 0.5 c.c., severe infections. Although the experiments present clinching evidence of the specific nature of the aggressin of neutral filtrates it might be well to determine whether the metabolic products of organisms other than *B. welchii* might exert a nonspecific aggressive action.

We chose at random 2 different organisms, *Proteus vulgaris* and the cholera vibrio. These organisms were grown for 18 hours in 0.2% glucose broth, the growth, which was heavy, was centrifugated and the supernatant, after neutralization, was filtered as in the case of the aggressin of *B. welchii*. A neutral culture filtrate of *B. welchii* of known aggressive power was used as a control. The organisms were, as usual, washed cultures of the 617d strain.

TABLE 8  
ATTEMPT AT NONSPECIFIC AGGRESSIVE ACTION

Exper.	Number	C.c. of Washed Organisms	C.c. of Welch Filtrate	C.c. of Cholera Filtrate	C.c. of Proteus Filtrate	Result
A	1	0.1	0.5	...	...	+ 17½ hours; gas, edema
	2	0.05	0.5	...	...	+ 18 hours; gas, edema
	3	0.01	0.5	...	...	+ 17 hours; gas, edema
	4	0.001	0.5	...	...	+ 23 hours; gas, edema
B	1	0.1	...	0.5	...	0
	2	0.05	...	0.5	...	Slight edema
	3	0.01	...	0.5	...	0
	4	0.001	...	0.5	...	0
C	1	0.1	...	...	0.5	Slight edema
	2	0.05	...	...	0.5	0
	3	0.01	...	...	0.5	0
	4	0.001	...	...	0.5	0

*Experiment A.*—There was added 0.5 c.c. of neutral filtrate to 1 c.c. of 1:10, 20, 100, and 1,000 c.c. of the washed organism. The mixtures were shaken thoroughly and injected at once.

*Experiment B.*—Like A, except that 0.5 c.c. of the cholera filtrate was used in each case.

*Experiment C.*—Like A, but proteus filtrate was used.

The suspensions of washed organisms in all 3 experiments came from the same pool. The injections were made intramuscularly into guinea-pigs which weighed 250 gm. The results are given in Table 8.

The neutral filtrates of 2 organisms other than *B. welchii* failed to exert an appreciable aggressive effect. The test is a rigid one, since the 0.1 c.c. dose of washed organisms is almost sufficient to kill when no neutral filtrate is added. And this brings up the question as to how washed organisms without the aid of the supernatant fluid are able to



cause infection. The minimal infecting dose for twice washed *B. welchii*, without the aggressin, varies between 0.1 and 1.0 c.c. It would seem apparent then, that the organism cannot be called a pure saprophyte, since beyond a certain dose it is able to infect without the aid of the aggressive filtrates. On the other hand, it is possible that some of the toxin is closely bound to the cell wall and in this way imparts a necrotic action to the bacilli themselves.

We have tried to approach this question by the testing of the comparative virulence of organisms washed twice and 5 times with large volumes of 0.85% NaCl solution. We found that the organisms after 5 washings were fully as virulent as those that had been washed twice. It is apparent then, that even the most thorough washing is unable to remove or destroy the ability of large quantities of organisms to infect. It is highly probable, though not proven, that we are dealing here with a preparatory necrotic action due to toxin closely bound to the bacterial cell bodies.

#### THE EFFECT OF NEUTRAL FILTRATES ON PHAGOCYTOSIS

The "aggressins" of Bail are supposed to operate through the negative chemotactic effect they exert on leukocytes. While we were quite certain that the major part of the aggressive effect of our neutralized cultures filtrates was due to a necrotic action, it was possible that these fluids might exert an injurious effect on the white blood cells. The idea suggested itself because of the frequently observed marked absence of phagocytes in the exudates of gas bacillus infections. We thought it best to approach this question by a study of infections produced by intraperitoneal inoculation, since it was likely that the mobilization of phagocytes would be more rapidly accomplished at this point than in muscular tissue.

Repeated experiments on this problem failed to disclose any decisive information. Occasionally large masses of phagocytes were found in the exudates of infections after intraperitoneal inoculation of broth cultures. At other times but few phagocytes appeared, the peritoneal fluid swarmed with rapidly multiplying organisms, and the animal died in a few hours. The use of the washed organisms and filtrates rendered equally indecisive results. Comparative tests were made on the effects of acid and neutral culture supernatant on the phagocytosis of *B. welchii* after intraperitoneal inoculation. The acid of the cultures did not seem to check phagocytosis, nor did the neutralized filtrate, as

compared with plain broth have any effect. It must be concluded that by far the most important aggressive activity of the toxin resides in its necrotic effect.

#### DISCUSSION

The foregoing experiments throw light on the mechanism of infections by *B. welchii* in animals and by inference on the method of extension of the infection in the human body. In the latter case the primary conditions for infection are established by the tissue damage resulting from the wound. The spores introduced at this time are able to germinate, and having passed into the vegetative stage, find in the injured muscular tissue an admirable medium for the production of the specific toxic substance. This substance, with its diffusibility aided by the outpouring of edema fluid that invariably accompanies infection, is able rapidly to necrose further tissue and so furnish new medium for the growth of the organisms. Finally an area of necrotic tissue sufficient to furnish medium for the growth of an enormous number of organisms is produced. These then are able to produce enough toxin to bring about toxemic death.

We have repeatedly tried to produce active immunity in rabbits and guinea-pigs by the injection of killed washed organisms. Such attempts have invariably failed, as have those to prevent infection by the use of a bacteriolytic serum. It seems to be very difficult to bring about any degree of opsonic or lytic immunity against *B. welchii*.

But in the light of the data brought forward in the preceding pages, the outlook for the prevention and treatment of gas gangrene is very bright. By the use of the specific antitoxin, which has been obtained in high potency by Bull, the all important aggressive factor is capable of neutralization. The only possibility standing in the way of remarkable results even in treatment is that of a comparatively greater affinity of the toxin for muscle than for antitoxin.

The resistance of the human body to *B. welchii* is very high and the primary invasion is accomplished by spores. It should be possible then, by proper methods of infiltration, to prevent infections from starting. And experiments just published by Bull,<sup>5</sup> indicate that this explanation of the method of infection is probably the correct one, since this investigator has been able in guinea-pigs to cure infections by antitoxin even after these had made great headway.

<sup>5</sup> Jour. Exper. Med., 1917, 26, p. 603.

## SUMMARY AND CONCLUSIONS

The acidity of cultures of *B. welchii* is not the prime cause of their ability to produce experimental infections. Neutralized cultures produce fatal infections in guinea-pigs in practically the same amounts as do acid cultures.

Bacilli, removed from broth cultures by centrifugation and subsequently washed with large volumes of 0.85% NaCl, are far less infectious than equal numbers of organisms not separated from the medium in which they have grown.

The virulence of washed organisms is increased at least 10,000 fold by the simultaneous injection of nonlethal amounts of neutralized culture filtrate.

This aggressive activity of the culture filtrate is destroyed by heating to 70 C. for 30 minutes, and by the addition of the specific Welch antitoxin. It must be concluded from this that the aggressin of the filtrate and the toxin are identical.

This conclusion is strengthened by the fact that sublethal amounts of toxin made by the muscle culture method show a similar aggressive effect, which is likewise neutralized by the addition of antitoxin.

Nonspecific culture filtrates from cholera and proteus cultures do not increase the virulence of washed bacilli.

The aggressive substance (toxin) seems to act by reason of its necrotic effect and not by a negative chemotactic influence on leukocytes.

These experiments throw light on the mechanism of experimental infection and on the method of extension of that in the human body.



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Annex



